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THE UNIVERSITY OF ALBERTA

ORGANIC ACIDS IN DAIRY PRODUCTS

by

Marija Škerlj

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

Food Chemistry

Department of Food Science

EDMONTON, ALBERTA

Fall 1986

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(SIGNED) Marija Škerlj.....

PERMANENT ADDRESS:

Linhartova 84.....

61000 Ljubljana.....

Yugoslavia.....

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19

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **ORGANIC ACIDS IN DAIRY PRODUCTS** submitted by **Marija Škerlj** in partial fulfilment of the requirements for the degree of **Master of Science** in **Food Chemistry.**

.....

Supervisor

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.....

Date....

July 14th 1986

Dedicated to my parents

ABSTRACT

Using an Aminex HPX-87H liquid chromatographic column at 65°C with 0.02 N and 0.0045 N H_2SO_4 as mobile phase and UV detection at both 220 and 275 nm, organic acids were quantified in dairy products.

Citric, orotic, pyruvic, succinic, uric, formic, acetic, propionic, butyric and hippuric acids were determined.

Samples were collected from each stage of the production process, starting with the raw material (milk) to the end-product. The following commercially-made products were analysed: raw whole and skim milk, partly skimmed (2% BF) and homogenised milk, whipping and cereal cream, buttermilk and skim milk powder, cultured buttermilk, sour cream, yogurt, and cheeses (Camembert, cottage, Cheddar, pressed Mozzarella, stretched Mozzarella, Quark and Ricotta). A series of cheeses imported from Denmark, France, the Netherlands and Switzerland were also analysed.

The results of succinic, uric, formic, acetic and propionic acid correlated with the moisture content of the dairy products or with the amount of whey retained by curd in processing. On the other hand, orotic, pyruvic, lactic, succinic and hippuric acids, the levels of which reflected the microbial activity rather than the amount of whey present in the dairy product, did not reveal such a correlation.

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1. INTRODUCTION

Organic acids are important constituents of food products because they influence flavour, stability and keeping quality of foods. They are an index of maturity, ripeness or microbial deterioration of some foods as well as an indicator of the fermentation stages or other processing operations. The identification and quantification of organic acids is required by the food industry, since it is useful for understanding the textural and flavouring characteristics of many food products.

Dairy products are produced from the same raw ingredient, milk, which is subjected to a variety of additives, preservatives, vitamins and chemical and/or biological processes. Organic acids in dairy products are the result of the normal metabolic processes in the animal, hydrolysis of butterfat, desired action of bacterial cultures or microbial deterioration, as well as a direct addition of acids when required by the process. Data on the organic acids in dairy products are valuable in the dairy industry for quality control purposes and monitoring the processing, fermentation and ripening stages of the products.

Conventional methods for organic acid analysis are time-consuming. Chromatographic methods have been developed for determining organic acids in biological and dairy product samples (Palmer and List, 1973; Marsili *et al.*, 1981). Considering the speed and simplicity of both sample

preparation and determination and quantification of the acids in dairy products, high pressure liquid chromatography can replace many of these traditional techniques.

Based on the current knowledge of high pressure liquid chromatography, this study provides information on the amount of organic acids in dairy products, starting with the raw material through the processing stages and up to the end-product. As well, it follows the fermentation and ripening stages of fermented dairy products and cheeses.

2. LITERATURE REVIEW

2.1 Microflora of Dairy Products Involved in Production of Some Organic Acids

The amount of organic acids present in various fermented dairy products are dependent on the starter cultures used in the manufacture. The role of starter cultures can be summarized as follows:

- production of lactic and/or other acids
- production of volatile flavour compounds
- proteolytic and/or lipolytic activity
- production of other compounds (e.g., alcohol)
- prevention of growth of pathogens and many spoilage organisms (Tamine, 1981).

Most of the lactic acid bacteria used in the dairy industry belong to the genera *Streptococcus*, *Lactobacillus* and *Leuconostoc*.

Streptococcus bacteria are Gram-positive and spherical or ovoid in shape. They are homofermentative, producing only L(+)-lactic acid from glucose. With the exception of *S. thermophilus* (thermoduric), they are classified as mesophilic bacteria (optimum temperature: 20-30°C). *S. lactis* and *S. cremoris* are very often used together in the cheese industry. They produce lactic acid with very small amounts of secondary products (acetic acid, alcohol, carbon dioxide). They are also weakly proteolytic and lipolytic. *S. lactis* sub-sp. *diacetylactis* can also metabolise citrates to

form diacetyl and CO_2. It is a very important aroma-producing organism. *S. thermophilus* is a thermophilic (optimum temperature 37-45°C) organism used in yogurt and cheese making when production of acid is desired early in the vat and press. It is used in symbiosis with *Lactobacillus bulgaricus*, which produces lactic acid at a much greater rate late in incubation.

Leuconostoc bacteria are Gram-positive and spherical or ovoid in shape, and ferment glucose heterofermentatively. Besides D(-)-lactic acid, they are capable of producing carbon dioxide and aroma compounds (e.g., ethanol and acetic acid). Important aroma-producing organisms in the dairy industry are mesophilic *L. cremoris* (previously known as *L. citrovorum*) and *L. dextranicum*. They are capable of breakdown of citrate into oxalacetate and acetate. Oxalacetate releases carbon dioxide and pyruvate is formed. When this pyruvate is in excess of that required for lactate production, it is removed from the cell after conversion to other substances (diacetyl). Carbon dioxide is an important metabolic product, required for the "eye" formation of Swiss cheeses.

Lactobacilli are Gram-positive and can be divided into three main categories. *Thermobacteria* and *Streptobacteria* are homofermentative thermophilic bacteria, from which many species are used in the dairy industry. *Betabacteria* are homofermentative and are not important as dairy starters. *Lactobacilli* are thermophilic, rod-shaped, lactic acid-

producing organisms. *L. bulgaricus* produces D(-)-lactic acid and is, as previously mentioned, used symbiotically with *S. thermophilus*. *L. lactis*, which produces D(-)-lactic acid, and *L. helveticus*, which produces DL-lactic acid, are the main starters for Swiss-type cheeses and other cheeses with high make temperatures. *L. acidophilus*, which produces DL-lactic acid, *L. casei*, which produces L(+)-lactic acid in excess of D(-)-lactic acid, and *L. plantarum*, which produces DL lactic acid, are also used as dairy culture starters.

Other bacteria are sometimes incorporated into a dairy starter culture. These include *S. faecium* (modified cheddar cheese); *Brevibacterium linens* (Brick and Limburger cheeses); *Propionibacterium freudenreichii* sub-sp. *shermanii*, used in Swiss cheese varieties (Emmental, Gruyère, Appenzell), mainly for its ability to excrete metabolites which produce large gas holes in the cheese during the curing period; and *Bifidobacterium bifidium* (Bioghurt).

Moulds are mainly used for the manufacture of some semi-soft cheese varieties. Their roles are:

- to enhance the flavor and aroma
- to modify the body and texture of the curd

According to their colour and growth characteristics, they can be divided into two types (Nelson and Richardson, 1967). The white moulds (*Penicillium camemberti*, *P. caseiocolum*, and *P. candidum*) grow externally on the cheese (e.g., Camembert and Brie). The blue mould (*P. roqueforti*) grows

internally in the cheese (e.g., Roquefort, Blue Stilton, Danish Blue, Gorgonzola and Mycella). The *Penicillium* genera are rich in proteolytic and lipolytic enzymes and their activities lead to free amines, amino acids, carbonyls and fatty acids.

Yeasts in milk result in alcohol fermentation (Kefir and Kumiss).

2.1.1 Cultured products

Milk can be fermented in a variety of products, each different due to a controlled manufacturing environment which induces the organisms to impart different flavours to the finished products (Marshall and Law, 1984). The manufacture of cultured dairy products represents the second most important fermentation industry (next to alcoholic beverages), accounting for approximately 20% of all fermented products produced world-wide in 1978 (Law, 1982).

Natural yogurt is the end product of a symbiotic culture of *S. thermophilus* and *L. bulgaricus* growing at temperatures in the range of 35-45°C. A proportion of 1:1 of "cocci" and "rods" is considered to be optimum for flavour and texture production (Oberman, 1985). *Streptococci* grow faster at the beginning of fermentation, which results in accumulation of lactic and acetic acids, acetaldehyde, diacetyl and formic acid. That stimulates the growth of *Lactobacilli*, which have a stronger proteolytic activity. By liberating amino acids from milk proteins, they stimulate

the growth of *S. thermophilus*.

Cultured buttermilk requires a slower rate of acid production and a longer incubation time than yogurt. Milk is fermented with selected strains of acid-producing *S. lactis* and/or *S. cremoris* in combination with citric-acid fermenting (flavour-producing) *L. cremoris*. Buttermilk is produced at 21-24°C to retain the balance of both types of strains. At higher temperatures, the product is lacking in aroma bacteria and the acid producers multiply at much faster rates. The flavour of buttermilk is associated with the lactic acid produced, but traces of acetic acid, formic acid, alcohol, diacetyl and carbon dioxide are present.

Sour cream is manufactured in a manner similar to buttermilk, and the same strains of starter bacteria are used. The proper balance between the acid and aroma producers is necessary (Vedamuthu, 1982).

According to Law (1984), cheeses can be grouped according to (1) moisture content and (2) the complexity of their microflora:

Soft cheeses: moisture content 50-80%

- in the case of unripened cheeses (e.g., cottage cheese), a simple mesophilic lactic acid bacterial flora
- in the case of ripened cheeses (e.g., Camembert), a simple basic flora and surface mould growth

Semihard cheeses: mesophilic starter and a more complex manufacturing process; moisture content 45%

- internally salted
- immersion in brine and subsequent rubbing with salt

Hard cheeses: moisture content less than 40%

- relatively simple microflora (e.g., Cheddar); mesophilic starters used as in soft and semi-hard cheeses
- inoculated with mould spores (e.g., Danablue, Roquefort)

Swiss varieties: thermophilic and propionic acid-producing bacteria (e.g., Emmental and Gruyère)

Italian hard cheeses: mammalian lipases are used to develop strong fatty acid flavours (Law, 1982).

2.1.2 Unripened cheeses

Cottage cheese is a low acid, soft curd product. Its properties are directly attributed to growth and metabolism of the starter culture introduced into the milk (*S. lactis*, *S. cremoris* and *L. cremoris*). The first two bacteria lack citrate permease and produce only enough pyruvate to balance the need for NAD^+ regeneration in the production of lactic acid. On the other hand, *L. cremoris*, through citrate metabolism, allows formation of aroma compounds besides lactic acid.

Quark is manufactured with the addition of *S. lactis*, *S. cremoris* and *S. diacetylactis*. The latter has a function similar to that of *L. cremoris* in cottage cheese. It uses the pyruvate from citrate metabolism and the cellular

pyruvate pool to form acetyl-CoA with the excess lactic acid production. It forms carbon dioxide as well as aroma compounds (diacetyl).

Ricotta is an unripened whey cheese. The acid and heat coagulate and precipitate the proteins. The cheese does not ripen like natural cheese as it has no lactic acid bacteria present.

2.1.3 Ripened cheeses

Feta cheese is a white brined cheese, ripened for a short time so that the typical microflora derived from the starters can develop. An acid coagulum is formed with cultures of *S. lactis* and *S. cremoris*. The cheese is soft, salty and the texture is close but full of mechanical openings so the curd is not pressed.

Cheddar cheese needs a long storage time to develop full flavour, like other ripened cheeses. Lactic acid has a stabilizing effect on cheese because of its antibacterial properties and its lowering of the redox potential and pH. For that reason, enzymatic reactions proceed slowly. The starter organism used in cheddar cheese is *S. cremoris* (multiple strain). Normally homofermentative starters can, under certain conditions (aeration, restricted growth), produce alternatives to lactate from pyruvate (formic acid, acetic acid, ethanol, diacetyl, and acetaldehyde [Law, 1982]). Secondary lactic acid bacteria (*Lactobacilli*, *Pediococci*) are also responsible for heterofermentative

fermentation of lactose, which causes defects in cheese.

The manufacture of Swiss cheeses (Emmental, Gruyère, Appenzell) involves a high-temperature cooking stage which only thermophilic starters can survive. *S. thermophilus* and *L. helveticus* produce lactic acid at the early stage, and some acetic acid may be produced. This initial lactic fermentation by starters is followed by propionic acid fermentation by *Propionibacterium shermanii*, which results in production of carbon dioxide (eye formation) and propionic and acetic acid. *S. thermophilus* multiply first, while *L. helveticus* produce lactic acid later in the production process (after 8 hours). During the curing period the proteolytic action of the *Lactobacilli* ripens the cheese and breaks down the casein. Only after that stage do *Propionibacteria* begin to develop. Gruyère develops additional flavour due to growth of a surface flora (lactate-utilizing yeasts and, when the surface pH nears 6, *Brevibacterium linens* can also grow as a secondary flora).

Danish cheeses with "eye holes" (Samsoe and Havarti) are similar to the Swiss cheeses.

Port Salut is a semi-soft cheese with no eye holes. *S. lactis* and *S. thermophilus* produce lactic acid during manufacture. As they die out, the curd undergoes proteolytic and lipolytic changes associated with enzymes from the starter culture and rennet.

Edam and Gouda are semi-hard, close-textured cheeses with no "eye holes". A few small eyes are permitted in Gouda

but not in Edam. Acid production is restricted by replacing a portion of whey with water. This reduces the lactose content, increases the pH and results in a plastic body and close texture. Bacteria used are *S. cremoris* var. *hollandicus*, *S. lactis* and *S. diacetylactis*. The changes during ripening are similar to the hard-pressed cheeses. Casein is broken down by enzymes from the rennet and by proteinases from the starter culture. Gouda has a lower moisture content and can be matured longer than Edam. This allows more protein breakdown and more flavour.

Pressed Mozzarella is produced in a way similar to that of Cheddar cheese with *Streptococcus* culture.

Stretched Mozzarella is a Pasta Filata cheese. The manufacturing process includes a pulling stage, which gives a cheese of characteristic plasticity (Chapman and Sharpe, 1981). *S. lactis*, *L. bulgaricus* and *S. thermophilus* are used as starter cultures. Rapid acid production is required in the manufacture and the ripening period is short or non-existent.

2.1.4 Mould-ripened cheeses

In blue-veined cheeses (Roquefort, Danablue), the curd is inoculated with blue-green *Penicillium* moulds (*P. roqueforti*). The cheese is "spiked" to admit air so that the spores can germinate and the mould can spread throughout the inside of the cheese. That process is aided by gas-forming *Leuconostoc* bacteria that produce an open texture in the

cheese. Mesophilic lactic acid starters are used (*S. lactis*, *S. cremoris*). The *Penicillium* has a strong lipolytic and proteolytic activity. Blue-veined cheeses have a surface slime containing moulds, yeasts and micrococci.

Surface-mould cheeses (Camembert, Brie, Marcillat) are also prepared with mesophilic lactic starters (*S. lactis*, *S. cremoris*) which produce acid at a slower rate than that required for Cheddar cheese. The cheeses are inoculated with white moulds (*P. candidum*, *P. camemberti*, or *P. caseicolum*), which have strong lipolytic and proteolytic activities. *Leuconostoc cremoris* and/or *S. diacetylactis* can be used for production of carbon dioxide and aroma compounds.

2.2 Review of the Methods of Analysis

2.2.1 Determination of organic acids by methods other than liquid chromatography

2.2.1.1 Hippuric acid

Hippuric acid (benzoyl glycine) is the form in which dairy cows and other herbivorous animals dispose of toxic benzoic acid from their bodies. There are considerable variations in the acid level depending on feed composition. Hippuric acid in the urine of dairy cows varies from 0.01 to 4.15%, with an average of 1.17% (Healy, 1912).

The presence of hippuric acid in milk, as a constituent of the nonprotein-N fraction, was confimed by Patton (1953), who also developed a method of quantification. This method

consisted of continuous ether extraction of tryptic digested skim milk and determination of N in the extract by the Kjeldahl method. Prior digestion of protein with trypsin was required to convert the protein-N to nonprotein-N which, after acidification with 70% sulfuric acid, renders hippuric acid more readily extractable with ethyl ether. Continuous extraction for a period of 50 hr was needed to recover hippuric acid from its aqueous solution. Patton (1953) found the hippuric acid content of skim milk to range over a six month period from 3.8 to 6.4 mg, averaging 5.1 mg/100 ml, with no clear cut seasonal trend observed.

2.2.1.2 Lactic acid

Lactic acid determination in milk and other dairy products, which involves the metabolism of the lactic acid bacteria, has long been a research topic. Lactic acid is present in all fermented dairy products and is produced by both homo- and heterofermentative microorganisms. The acid exists in L(+)- and D(-)-forms as well as D,L-racemic mixture.

Both optical isomers of lactic acid are absorbed in the human intestinal tract, although the rate of metabolism of the D(-)-isomer is much lower than that of L(+)-acid (Alm, 1982). Lactic acid affects calcium resorption. Feeding assays with rats demonstrated that calcium retention is higher when rations contain fermented milk. When a high level of D(-)-lactic acid was incorporated into the diet, calcium was excreted in the urine. In addition, the

D(-)-form caused acidosis and reduced cell metabolism. Hence, dairy products with a high content of D(-)-lactic acid are not recommended in infant nutrition.

Lactic acid determination by titratable acidity, an indirect method, has many limitations. Direct methods are of greater value. One such method involves lactic acid oxidation to acetaldehyde and its stripping into a bisulfite solution, followed by titration of the bound bisulfite with iodine (Troy and Sharp, 1935).

Other methods involve extraction of lactic acid with ethyl ether from a protein-free filtrate, and development of its yellow-colored complex with ferric chloride (Hillig, 1942a,b; Ling, 1951; Steinsholt and Calbert, 1960). Another colorimetric method of lactic acid determination was proposed by Heinemann (1940). It involves lactic acid oxidation by sulfuric acid to acetaldehyde, followed by condensation of the aldehyde with veratrole. Instead of veratrole, reaction with p-hydroxydiphenyl was proposed by Barber and Summerson (1941). In the latter method both protein and glucose had first to be removed by copper hydroxide/calcium hydroxide solution. This method was widely adopted for milk and other dairy products (Davidson, 1948; see also Lawrence, 1975). Davidson found that the lactic acid content of fresh raw milk was 1.5-2.0 mg/100 ml, while fresh milk powder was 20-21 mg/100 g. The lactic acid contents of commercial samples of whole and skim milk powders exceeded 1,000 mg percent.

Rapid determination of lactic acid in whey by gas chromatography was developed by Gray (1976). The whey sample was freeze-dried and the lactic acid in the dried sample was esterified with 14% boron trifluoride in butanol. Separation and quantification of the ester was achieved on a 12% diethyleneglycol succinate (DEGS) column at 130°C using nitrogen as carrier gas. In routine analysis 5 ml whey was freeze-dried, nonanoic (pelargonic) acid was added as an internal standard, and then lactic acid was esterified by refluxing in butanol for 10 min. The ester thus formed was transferred from the salt-saturated aqueous phase into a hexane layer and, without further purification, 2-3 µl was injected into the gas chromatograph. The recovery test for lactate added to whey yielded 97.70±5.1%; the correlation coefficient between weights of lactate added and recovered was 0.9970. Thus, this method was not only fast but accurate. Moreover, it did not require deproteination of the whey prior to esterification. The method is used as a routine procedure for measuring lactate in whey and skim milk powder and as an index for the quality of milk selected for manufacturing milk powder.

Steffen (1971) provided reliable methods for determination of total lactic acid and its optical isomers in cheese and milk. Total lactic acid was estimated by Ling's (1951) colorimetric method using ferric chloride, or by the sum of the L(+)- and D(-)-isomers. The latter was determined by reduction of the respective isomers to pyruvic

acid by L(+)- and D(-)-lactate dehydrogenase in the presence of NAD and by following the absorbance of NADH at 366 nm. A quantitative conversion of lactic to pyruvic acid was ensured by the addition to the reaction mixture of L-glutamate, glutamate-pyruvate transaminase and hydrazine. The enzymatic method was highly specific and accurate, with a sensitivity of 4-5 µm of lactic acid/g cheese or milk.

Using the enzymatic method described above, Steffen *et al.* (1973) determined the optical configuration of lactic acid produced by 21 strains of lactic acid bacteria. All *Streptococcus* strains tested (*S. lactis*, *S. cremoris*, *S. thermophilus* and *S. faecalis*) and *Lactobacillus casei* strains assayed produced more than 92% L(+)-lactic acid. On the other hand, *L. bulgaricus* and *L. lactis* strains produced only D(-)-isomer. *L. acidophilus* and *L. helveticus* provided both isomers, with L(+)-acid being 60-70%. As proven by the authors, the configuration of the lactic acid formed was not affected by incubation time or temperature, nor by the pH or composition of the incubation media. Thus, the lactic acid configuration in some dairy products assayed (yogurt, cultured cream, quarg, buttermilk, cottage cheese, Emmental, Gruyère) had a higher amount of L(+)-lactic acid isomer that simply and reliably reflected the strains of lactic acid bacteria used.

Alm (1982) investigated the formation of lactic acid and its L(+)- and D(-)-isomers in some fermented dairy products made in Sweden. The author applied the enzymatic

method of Steffen (1971) and Steffen *et al.* (1973). The highest amount of total lactic acid, 1.2%, was in yogurt. This yogurt was produced from pasteurized milk inoculated with a mixture of *L. bulgaricus* and *S. thermophilus*. Next in lactic acid content were: cultured buttermilk, 0.76%; kefir (inoculated with a mixture of kefir grains), 0.71%; acidophilus milk, 0.69%; bifidus milk, 0.62%; and ropy milk, 0.50%. Lactic acid was not detected in regular milk.

Alm (1982) also reported that L(+)-lactic acid was the predominant isomer in all dairy products assayed, except in yogurt, in which about 40% of the total acid was present as D(-)-lactic acid. In yogurt with fermentation accomplished in 3-4 hr there was an exponential increase of L(+)- and a lower but steady linear increase in D(-)-lactic acid, which levelled off at about 40%.

In acidophilus milk inoculated with *L. acidophilus* (NCDO 1748), the content of D(-)-acid gradually increased during fermentation, reaching 100 mg/100 g after 24 hr and 150 mg/100 g after 40 hr of incubation. At the end up to 10% of the total lactic acid was in D(-)-form. Similar observations were found with bifidus milk (inoculated by *Bifidobacterium bifidum*; (*L. bifidus*, NCDO 11863) and with ropy milk (inoculated with a mixture of starter culture of *S. lactis* var. *longi* and *Leuconostoc cremoris*).

Lastly, Alm (1982) concluded that about 20-50% of the lactose present in milk is fermentable. That amounts to 0.6-1.2% of lactic acid in dairy products, a level still

much lower than the 2.7% of lactic acid tolerable to *Lactobacilli*. Also of interest was the conclusion that L(+)-lactic acid is the predominant metabolite in milk fermentation, which thus matches the configuration of the lactic acid produced by the human body.

2.2.1.3 Orotic acid

Orotic acid has been found to be prevalent in milk of ruminants rather than nonruminants. Levels reported for milk and some dairy products conflicted due to earlier bioassay procedures that also measured other pyrimidine nucleotides.

Chemical spectrophotometric methods introduced by Tsugo *et al.* (1966) and Adachi *et al.* (1963) involved the conversion of orotic acid to barbituric acid and complexation of the latter with p-dimethylamino benzaldehyde.

The mechanism of the above reaction is as follows: orotic acid is brominated to 5,5'-dibromobarbituric acid; this is then reduced by ascorbic acid (debrominated) to barbituric acid. The latter, having an active methylene group, condenses with p-dimethylamino benzaldehyde, providing the chromogen 5-(dimethylaminobenzylidene) barbituric acid. In the reduction step it was found that ascorbic acid gives better results than some thiol reducing compounds.

Larson and Hegarty (1979) applied this chemical method for milk analysis but included a prior deproteination step using 2% trichloroacetic acid. The orotic acid was then

determined in the filtrate after being converted to barbituric acid. Particulars of this procedure are: milk (1 ml) is diluted with water to 5 ml, then chilled and treated with 0.2 ml cold 50% trichloroacetic acid (TCA; final concentration 2%). The sample is left overnight at 4°C in order to complete protein precipitation. The mixture is then washed twice with 0.4 ml cold 2% TCA. The combined filtrate and washings are analyzed. An aliquot of 0.5 ml is assayed according to the revised method of Tsugo *et al.* (1966).

The above study reported levels of orotic acid to be 88±19 μg/ml in dairy cow milk and slightly less in beef cow milk, 76±17 μg/ml, all mid-lactation milk. However, dairy colostrum milk collected the first day had only 21±7 μg/ml of orotic acid. Other dairy products had orotic acid contents which appeared to depend on the amount of soluble whey retained and/or the extent of fermentation of the product. Thus, cottage cheese had a mean of 39 μg/g product; cream cheese 40; evaporated or dried skim milk 165 and 802, respectively; and yogurt 66; while aged cheeses had the lowest content, 16, or for Roquefort, 15.

As reviewed by Chen and Larson (1971), orotic acid biosynthesis starts by condensation of carbonyl phosphate with aspartic acid. The condensation product, by water elimination, provides dihydroorotic acid which, in the presence of NAD, is then oxidized to orotic acid. These biosynthetic steps coincide with a normal pathway of pyrimidine biosynthesis. Orotic acid does not accumulate, but is

further converted to uridine or cytidine phosphates and to correspondig deoxyribonucleotides.

The above authors analyzed three enzymes in bovine mammary tissue: dihydroorotic acid dehydrogenase, orotidine-5'-phosphate pyrophosphorylase and orotidine-5'-phosphate decarboxylase, i.e. the three major enzymes involved in orotic acid biosynthesis. The enzyme activities reported were adequate to justify the orotic acid accumulation in bovine milk. By applying the method of Adachi *et al.* (1963), they found orotic acid contents in individual cow's milk of 68.4, 63.9, 58.5 and 85.8 µg/ml. For milk of pooled herds, results were 72.1, 74.0, 70.8 and 59.2 µg/ml. For a Guernsey cow in the first week of lactation the authors found 42.6 µg/ml and, for the same cow's market milk, 71.2, 68.9, 67.8 and 71.4 µg/ml. It was of interest that the 74.0 µg/ml result obtained by applying the Adachi *et al.* (1963) method corresponded to the 70.0 µg/ml obtained by the enzymatic method of Rosenbloom and Seegmiller (1964).

2.2.1.4 Pyruvic acid

Zandstra and de Vries (1977) studied the pyruvate content of raw cow's milk and related its content to an index of milk bacteriological quality. The authors stated that pyruvic acid content does not appear to provide reliable information on the initial microbiological contamination of milk. However, the content of acid, as a central compound in bacterial metabolism, provided information on

the extent of glycolytic, proteolytic and lipolytic activities which occur in refrigerated milk.

The authors determined the pyruvate content via enzymatic reaction:

$$\text{Pyruvate} + NADH_2 \xrightarrow{\text{Lactate dehydrogenase}} \text{Lactate} + NAD$$

The decrease of $NADH_2$ concentration was proportional to the amount of pyruvate and was continuously recorded spectrophotometrically at 340 nm. The authors found that the pyruvate added to milk was recovered quantitatively with a deviation lower than 3% (n=8) and that the lowest detectable level of acid was 0.2 mg/l milk. The standard deviation of the pyruvate measurements was 0.08 mg/l, while the extinction coefficient for NADH, measured in a standard pyruvate solution, was found to equal that cited in the literature for pure NADH.

Marshall and Harmon (1978) automated the above enzymatic method for quality testing of milk. A Technicon Auto Analyzer II (Technicon Co., NY) was used, with sample combined with 7% sodium citrate and mixed with air bubbles. The pyruvate-containing sample was pumped together with reduced nicotinamide adenine dinucleotide and the enzyme lactate dehydrogenase, and the absorbance was recorded at 340 nm. This automated method gave the following results for pyruvic acid content: 0.47 mg/l for normal milk and 2.5 mg/l for fresh pasteurized milk. The pyruvate content of mastitis-affected raw milk was 1.34 mg/l. The increase in

pyruvate content was found to be the result of an increased number of somatic cells in the milk and/or bacteria. However, an increase in pyruvate content in milk was found with increased standard plate count only in mastitis-affected milk. These findings unequivocally supported the results reported earlier by Zandstra and de Vries (1977). In addition, Marshall and Harmon (1978) found that a pyruvate test is not a sufficiently reliable indicator for a psychrotrophic count because psychrotrophic bacteria metabolize pyruvate in the stationary phase of their growth when other energy sources are depleted. Lastly, the authors suggested pyruvate values of 2-2.5 mg/l as acceptable levels for good quality raw milk kept in bulk tanks.

Metabolites other than pyruvate were also related to an index of bacteriological quality of raw milk. Enzymatic breakdown of carbohydrates to lactate, fats to free fatty acids (FFA) and proteins to ammonia was related to milk quality by Suhren (1982). As emphasized by the author, pyruvic acid is the central metabolic product for the degradation of all three classes of these compounds. The acid content was followed by the enzymatic method of Zandstra and de Vries (1977) combined with automatization provided by an Auto Analyzer II system, developed by Technicon Co. (New York). Similarly, lactate determination was automated by the reverse reaction:

$$\text{Lactate} + \text{NAD} \xrightarrow{\text{Lactate dehydrogenase}} \text{Pyruvate} + \text{NADH}_2$$

The coupled reduction of NAD was measured at 340 nm.

Determination of ammonia was also automated. The determination involved its conversion to chloramine, which then reacts with phenol to provide a blue chromogen, indophenol, readily measured spectrophotometrically at 610 nm.

Lastly, Suhren (1981) measured the free fatty acid content, also by an automated method. The free fatty acids were extracted from milk with a mixture of isopropanol, heptane and sulfuric acid and then the color developed with phenol red in the organic phase was measured by auto analyzer.

The above study provided the following results: pyruvate content was 1.66 mg/kg (or ppm) milk at the farm; 2.05 mg/kg at the bulk truck tank level; and 2.54 mg/kg at the dairy plant. Corresponding lactate levels were 17.1, 24.6 and 26.8 mg/kg, respectively. The ammonia contents were 3.0-6.0 mg/kg for freshly-collected milk and 3.2-6.5 mg/kg for raw bulk milk in tank. Values above 8.5 mg/kg indicated inferior milk quality. FFA content of bulk herd milk was 0.4-0.7 milliequivalents/l.

The indicators of analysis precision, the coefficients of variation, were calculated from the analysis of duplicate samples and were as follows: 1.73% for pyruvate; 3.22% for lactate; 1.03% for ammonia; and 2.13% for FFA. The recovery assay for pyruvate, lactate and ammonia was in the order of 100%, while for FFA it was a function of the chain length. Lower fatty acids had lower recoveries (C_4=67%), while

higher fatty acids (chain length ≥ 16) had recoveries of 99%.

As stated by Suhren (1981), refering to FR Germany, quality testing to assess payment for raw milk permits either colony counts or pyruvate determination, the methods having equal validity.

2.2.1.5 Other organic acids, with emphasis on free fatty acids

Separation and identification of sixteen biologically important organic acids by an initial separation on a silica gel column was introduced by Bulen *et al.* (1952). Mallinckrodt grade silicic acid, coarse particle size, was slurried with 0.5 N sulfuric acid and packed in a column about 5 cm by 1.2 cm i.d. The eluent consisted of 5, 15, 20, 25, 35 and 50% (v/v) n-butanol in chloroform and of 25% (v/v) n-butanol in benzene. Prior to use, each eluent was equilibrated against 0.5 N sulfuric acid. The acid fractions were collected and titrated by microburet with 0.01 N sodium hydroxide in the presence of phenol red as indicator. With the proper eluent schedule the elution sequence of acids was as follows: acetic, pyruvic, glutaric (plus fumaric and formic), lactic (plus succinic and α-ketoglutaric), trans-aconitic, oxalic (with glycolic and cis-aconitic), malic, citric, isocitric, and tartaric. Acids coeluted had to be separated by an additional treatment on a larger column. Thus, the mixture of fumaric, glutaric and formic acids was separated on a column 60 cm by 7 mm i.d., prepared as the first column from silica gel slurry, however, the

eluent was 20% n-butanol in chloroform. Alternatively, rechromatography of the acid mixtures could be done on the first column using 25% n-butanol in benzene. Separation on the first column was long, requiring about 9 hr. This method was initially suggested as an analytical tool for separation of organic acids from plant tissues. It was successfully applied for tomato and other fruits and green leaf tissues.

Based essentially on the above method, Harper *et al.* (1956) developed a rapid method for measuring the total free fatty acids (FFA) in milk. The authors pepared the chromatography column in two sections. The bottom section contained a slurry of 5 g dry silicic acid (Mallinckrodt) in 3 ml phosphate buffer pH 6.5 and 20 ml chloroform. The top section of the column contained the sample, 3 ml of milk acidified to pH 1.8-2.0 with 20% sulfuric acid, and 5 g of silica gel as in the bottom section. This mixture was ground and slurried in an excess of 5% n-butanol in chloroform. When the mixture was added to the column and 15 ml of effluent collected, all the free fatty acids were eluted. The effluent was titrated with methanolic 0.01 N KOH in the presence of phenol red as indicator. The results were expressed as acid degree (by definition the ml of 0.01 N base needed to neutralize 100 g fat/oil). Recoveries of acids were: butyric, 99%; caproic, 100%; capric, 93%; lauric, 94%; palmitic, 90%; and oleic, 96.0%. Reproducibility of the method was shown by an average mean deviation of ±4.05%. The fatty acids in milk could be separated in one fraction, using a single eluent, and determined by titration

within 15 min/sample. The method was also suitable for analysis of dry and condensed milk, ice cream and butter.

Ohren and Tuckey (1969) related cheddar cheese flavor to the balance of free fatty acids and acetate content. They applied silicic acid column chromatography and GLC as well as organoleptic evaluation. Total free fatty acids were determined by the method of Harper *et al.* (1956), and the content of acetate by separation of acetic acid on silica gel by the method of Bulen *et al.* (1952). Further separation of free fatty acids from other cheese lipids was achieved by the method of McCarthy and Duthie (1962).

The above authors found that the characteristic cheddar cheese flavor depended on the treatment of the milk received, and on its bacterial population. The flavor and physical properties of the cheese were superior when normal fat milk was used instead of low fat milk. But, the sum of free fatty acids and acetate was not dependent on the fat content of milk. Also, their findings agreed with common knowledge that cheeses with higher fat contents contain higher amounts of FFA and a lower level of acetate. They suggested that all compounds contributing to cheddar cheese flavor should be in balance and that acetic acid is the dominant volatile.

Thus, for optimum cheese quality, two criteria have to be satisfied. First, the sum of FFA and acetate at the end of the 180 days of ripening stage must be between 12 and 28 μmoles per g solids, and, second, the ratio of μmoles of FFA/μmoles of acetate must be between 0.55 and 1.0.

McCarthy and Duthie (1962) developed a quantitative method for the separation of free fatty acids from a mixture of various lipid classes. The method was of interest for the determination of butyric, valeric and some longer chain fatty acids. The lipid sample, dissolved in ethyl ether, was introduced on a column of silicic acid previously treated with isopropanolic KOH. Neutral lipids were eluted with ethyl ether. Free fatty acids were then separated by 2% formic acid in ethyl ether, followed by pure ethyl ether. During this separation, phospholipids were retained by the column and they did not affect the recovery of free fatty acids. Finally, phospholipids were removed using methanol as an eluent. However, the phospholipid fraction was contaminated with potassium salts. Hence, quantification of this fraction would necessitate isolation on an untreated silicic acid column.

Determination of individual free fatty acids in dairy products using GLC was introduced by Hankinson *et al.* (1958). Their method was based on GLC separation of formic up to octanoic acid, and collection of acids in aqueous traps, followed by acid titration.

Bills *et al.* (1963) reported the use of methyl esters rather than free fatty acids for GLC analysis. FFA were isolated from dairy fat by applying a basic anion exchange resin. The same resin was then used for direct esterification of the acids, followed by extraction of the esters with ethyl chloride. The extract was then cautiously concentrated to prevent losses of the more volatile, low-chain esters. In

some dairy products, such as fresh or rancid cream or butter, they confirmed in all samples the presence of $C_{4:0}$ to $C_{18:0}$ fatty acids in addition to C_{18} with one, two or three double bonds.

A valuable improvement of dairy product analysis using GLC was obtained by introduction of capillary columns. Palo and Hrivnak (1978) applied an initial step of analysis suggested by Morgan and Day (1965): stripping of the volatiles by an inert gas. The stripped volatiles were trapped in a chilled precolumn and, after heating, transferred to an analytical GLC column. However, they used not only analytical capillary coloumns but also capillary precolumns and a split less injection method.

Carl (1984) emphasized the superiority of open tubular capillary columns over packed GLC columns. Fused silica columns with crosslinked nonextractable stationary phases connected to an injection port with a split/split less mode provided a high degree of reliability in quantitation of free and/or methylated fatty acids.

2.2.2 Determination of organic acids by liquid chromatography

Palmer and List (1973) were among the first to describe the determination of organic acids in food by liquid chromatography using high efficiency anion exchange columns. These acids included Krebs' cycle acids (citric, isocitric, α-ketoglutaric, succinic, fumaric, malic and oxalacetic), some alicyclic acids such as quinic and shikimic acids, and

some other acids such as acetic, galacturonic, lactic, malonic, oxalic and tartaric. They also found that a precolumn injection system sharpens the acid peaks and permits the analysis of aqueous extracts with no pretreatment. A refractive index detector system provided continuous monitoring of the effluent.

The column packing consisted of Aminex A25 (formate form, Bio-Rad), a strong basic anion exchange resin. The eluent was Na-formate 1.0 N with a flow rate of 1-2 ml/min at a column temperature of 70°C.

The aqueous samples were injected via a sample loop into a precolumn system, 3.6 cm in length. The acids were retained at the top of the precolumn, while neutral and basic components of the extract were washed out by the pumping water. Then the precolumn was connected to the top of the separation column (75-90 cm length, 6 mm i.d.) and, with a second pump, Na-formate was pumped through the precolumn and then the main column.

An important feature of their method was the use of the precolumn, which allowed the total acidity to be increased to about 250 microequivalents. Then the acids were transferred to the main column in the form of a narrow band. This enabled an increase in both sensitivity of detection and acid resolution. The peaks were markedly sharpened at elevated temperatures. Temperatures above 70°C were avoided to prevent column deterioration, presumably due to loss of crosslinkages and conversion of quaternary ammonium to amino groups. Though the method provided excellent separation of

organic acids, the authors applied it only to a series of beverages of interest to the food industry.

Separation of citric acid cycle acids by liquid chromatography in an industrial quality control lab was also suggested by Turkelson and Richards (1978). Instead of using columns of acidified silica gel with n-butanol/chloroform or tert-amyl alcohol/chloroform gradients, these authors were among the first to suggest the use of strong cation exchange resins and dilute minerals acids as a mobile phase. Also, they were among the first to replace the laborious collection of numerous fractions and their titrimetric analysis or acid extraction and derivatization as a means of effluent monitoring, by using UV monitoring at 210 nm. By introducing ion exclusion and partition chromatography with continuous monitoring, they separated the citric cycle acids (cis-aconitic, α-ketoglutaric, oxaloacetic, citric, isocitric, malic, fumaric and succinic) in less than 30 min. The use of dilute mineral acid instead of water as a mobile phase suppressed the ionization of strong and moderately strong organic acids, and forced them to partition in the resin phase.

Aminex 50 W-X4 cation exchange resin (Bio-Rad) was packed in a glass 300 x 12.7 mm i.d. column fitted with a metal plunger. At a flow rate of 1 ml/min the retention time and peak intensities of the components were reproducible within 1 and 2%. As stated by the authors, the overriding factor in acid separation was clearly the pK's of the acids. When all variables were held constant, the stronger acid was

first to be eluted from the column. The detection limits varied: cis-aconitic had the lowest (<1 ppm) and succinic acid the highest limit (30 ppm).

Major organic acids in potato tuber, oxalic, citric, malic, fumaric and ascorbic, were quantitated by an ion exclusion LC method by Bushway *et al.* (1984). Tubers were extracted with 95% ethanol/water/conc. sulfuric acid (60:40:0.2) and the extract injected on an Aminex HPX-87 column. The acids were separated using 0.018 N sulfuric acid as a mobile phase at ambient temperature. A Bio-Rad column of 300 mm x 7.8 mm i.d., packed with resin of 8% cross-linkage and 9 μm particle size, was used. The eluting acids were monitored at 210 nm, except for ascorbic acid, which was monitored at 260 nm, and malic acid, in the presence of fructose, was determined at 220 or 230 nm.

Reverse phase HPLC requires lengthy ion-exchange column clean-ups, however, the ion exclusion method eliminates the need for such a clean-up step. This feature should be considered a major advantage for all methods based on ion exclusion column chromatography.

Sample preparation was also simplified in the above procedure. Tubers, 50 g (peeled or with skin), were blended for 5 min with 100 ml extracting solvent. The slurry was filtered through Whatman No. 2 filter paper and a 2 ml aliquot refiltered through a 0.45 μm Millipore filter. In order to avoid errors due to the presence of different compounds with identical retention times, the absorbance ratio method was used for organic acid peak identification.

Thus, by utilizing absorbance ratios of 210/220, 210/230 and 220/230 nm, the presence of succinic acid was confirmed only in a few tuber cultivars. Malic and ascorbic acids were coeluted. Hence, the latter acid was determined at 260 nm, at which malic acid had no absorbance. To quantify malic acid, the ascorbic acid had to be oxidized to its dehydro form. The retention time of this form was shifted between those of citric and malic acids.

Sugars, such as fructose, glucose and sucrose, present in higher levels in tubers stored at 3.3°C, were also eluted under the separation conditions applied. Only fructose interfered, since it shouldered the malic acid peak. The latter peak was quantitated at 220 and/or 230 nm instead of 210 nm.

Among the systems tested, the efficiency of the chosen system equalled that of acetonitrile/0.01 N sulfuric acid (20:80 v/v) or systems incorporating metaphosphoric acid. Of importance was the finding that 80% ethanol, which is used extensively as acid solvent, was only 65-75% as effective as the solvent selected by the above authors.

A very specific area which has been and is still currently well served through the use of high pressure liquid chromatography (HPLC) is the dairy industry. Size exclusion or gel permeation chromatography is applied to monitor cholesterol levels in cream cheese or butter. Specialty columns have been used for carbohydrate analysis in egg nog (fructose, glucose, sucrose and lactose), ice creams, sweet and condensed milk, flavored yogurts and other

feremented dairy products. By introduction of reverse phase columns, rapid analysis of β-carotene in butter or margarine has been achieved, while artificial colors such as tartrazine (FD&C Yellow No. 5) have been monitored by ion pairing mechanism.

Ascorbic acid content in dairy products is also monitored by HPLC. The radial PAK U Bondapak-NH_2 cartridge has been the column of choice. For this purpose the extraction solvent contained acetonitrile, which precipitates protein, and thiourea, and antioxidant, to protect the ascorbic acid from oxidation. HPLC is also used for analysis of vitamins such as B_6, A-palmitate, niacin and D.

HPLC is a powerful tool for controlling the quality of raw milk and fermented dairy products. This involves the separation of several organic acids, some of which are responsible for flavor and coagulation properties of the dairy products. This is especially the case with monitoring the lactic acid levels in dairy fermentation processes.

Analysis of organic acids in feremented whey and some commercial dairy drinks by HPLC was reported by Hamakawa *et al.* (1983). Using a strong cation Shodex Ionpac C-811 column and 0.1% (w/v) aqueous phosphoric acid as eluent, they separated a total of 13 acids with satisfactory resolution. In cheese whey and its fermented products and in skim milk they confirmed the presence of orotic, citric, pyruvic, succinic, lactic, uric, formic, acetic, propionic and hippuric acids. The eluates were monitored and

quantitated by their ultraviolet absorption at 220 and 275 nm.

A versatile HPLC method for the quantitative determination of lactic acid in a wide variety of foods and, particularly, fermented dairy products was reported by Ashoor and Wetty (1984). They used a 300 x 7.8 mm Aminex HPX-87 column in conjunction with a microguard ion exclusion cartridge. The mobile phase was 0.009 N sulfuric acid and the effluent absorption was monitored at 210 nm. The lactic acid standards and sample solutions contained 0.05% EDTA. Sample prepartion of liquids involved 10-20 ml sample, filtering through Whatman No. 1 paper, adding 5 ml of 1% EDTA to the filtrate, and adjusting the volume to 100 ml. Semisolids and solids were blended with water for 3 min, then filtered and centrifuged. The filtrate or supernatant was mixed with 5 ml 1% EDTA and the volume made up to 100 ml. The presence of EDTA masked the free metal ions in the sample preparations and thus maintained the column efficiency, yield reproducible results.

The 5.6 min retention time of EDTA was shorter than that of lactic acid (11.4 min) so it did not interfere in lactic acid quantification. By dividing the absolute retention time of lactic acid by that of EDTA, the relative retention time of lactic acid was obtained, and for all dairy samples it was 2.03±0.02. Recovery studies involved spiking the lactic acid peaks and amounted to 93.4±2.2 g/100 g for buttermilk, 95±3.4 for Swiss cheese and 97.0±2.9 for plain yogurt. With a coefficient of variation (%) varying

from 2.3 (buttermilk) to 4.9 (blue cheese), the lactic acid contents were found to be: buttermilk, 0.911±0.021 g/100 g; plain yogurt, 1.208±0.050; Swiss cheese 0.671±0.021; and blue cheese, 1.275±0.063. Based on these results, Ashoor and Wetty (1984) concluded that quantitative determination of lactic acid in dairy products is simple, rapid and specific and, moreover, accurate.

Determination of orotic acid and other carboxylic acids in milk and dairy products by liquid chromatography was the topic of investigation by Lavanchy and Steiger (1984) at the Federal Dairy Research Institute, Bern (Switzerland). Sample preparation involved 5 g of milk or cheese and its homogenization with 5 ml water and 20 ml acetonitrile, the latter used as a protein-coagulating agent. The suspension was centrifuged and an aliquot filtered (0.45 μm pore size Teflon filter) and then injected into the HPLC. The column used was Aminex HPX-87, 300 x 7.8 mm, at 50°C, with 1% (v/v) acetonitrile in 0.009 N sulfuric acid as eluent. The results reported for raw milk, yogurt and sour cream, in mg/kg, were as follows: orotic acid, 73.3 (75.5, 45.4); citric acid, 938 (725, 130); lactic acid, 91.5 (16,280, 9,215); uric acid, 16.5 (30.7, 15.4); acetic acid, 0 (110, 880); propionic acid, 0 (0, 175); butyric acid, 0 (0, 0); and hippuric acid, 36.6 (0, 0).

Preparative separation of milk fatty acid derivatives by HPLC, required for fatty acid structural analysis, was introduced by Christie *et al.* (1984). A reverse phase column was used. Aliphatic esters were monitored by

refractive index, and aromatic esters by UV detection.

In Christie *et al.*'s method the lipids were first extracted from milk with chloroform-methanol (2:1, v/v), then dissolved in acetonitrile. Transesterification followed in methanol, using 1 M K-methoxide as a catalyst, for 5 min. The reaction was terminated by addition of acetic acid. The esters were cleaned through a neutral alumina column prepared in a disposable Pasteur pipette, and a clarified aliquot was then injected onto the LC column. Benzyl esters were similarly prepared in benzyl alcohol solution.

The column system consisted of a 5 x 0.5 cm guard column and a 25 x 0.5 cm main column packed with Li Chromosorb 10 RP 18. Isocratic elution was applied using acetonitrile-water (95:5, v/v) at a flow rate of 1 ml/min. This provided reliable separation of methyl (benzyl) esters of fatty acids in a chain length order of 4:0, 6:0, 8:0, 10:0, 12:0, 16:1, 14:0, 18:1, 16:0, and 18:0. By using acetonitrile alone as eluent, better resolution was obtained and the separation time was only 20 min. However, methyl butyrate eluted with the reagent peak. Benzyl esters of the longer chain fatty acids eluted with poor resolution. In this case, to improve the resolution after the 14:0 acid, the flow rate was doubled to 2 ml/min.

Organic acid precolumn derivatization with p-bromophenacyl bromide, bromomethoxycoumarin or 9-antrhyldiazomethane has been employed. Also, postcolumn derivatization with dicyclohexylcarbodiimide, bromocresol purple or bromophenol blue has also been reported (Wada *et al.*, 1984).

Bromocresol purple, bromophenol blue or bromothymol blue were used as pH indicators, acting as postcolumn reagents. When an organic acid was eluted, there was a change in indicator color. This was monitored photometrically at the indicator absorbance maximum. Thus, Wada *et al.* (1984) designed a reaction system which included sample delivery and a bromothymol blue (BTB) reagent delivery pumps. After the organic acids were separated on a strong cation exchange resin, Shodex Ionpak (500 x 8.0 mm i.d.), the acids and the reagent were mixed in a postcolumn reaction coil and the color change monitored at 445 nm. The eluent was 3 mM perchloric acid, neutralized with 2 mM NaOH, while the molarity of the BTB reagent was 0.2 mM and it was dissolved in 15 mM Na_2HPO_4 + 2 mM NaOH. Such a system provided a sensitivity for lactic acid of close to 0.1 absorbance unit per 40 µg acid, or 0.2 absorbance unit for the same levels of citric, malic or succinic acids. A great asset of this method was the linearity of the calibration curves for the six tested organic acids, following the general equation $h = ax + b$; where h = peak height ($x10^{-3}$ absorbance), and x = µg sample. The factors a (b) for some acids were: citric, 4.250 (1.130); malic, 4.329 (2.408); succinic, 4.413 (2.598); lactic, 3.030 (1.339). Correlation coefficients were close to 1.0, reflecting the good linear relationship between the acid contents and peak heights.

Simple isocratic reverse phase and cation exchange LC methods have been successfully applied in clinical

biochemistry for screening organic acid disorder in neonates (newborns ranging from 1 day to 4 weeks of age) and infants. Through urine analysis, methylmalonic, propionic, isovaleric, lactic, glutaric or medium chain (C_6 to C_{10} dicarboxylic acids) acid ureas or β-ketothiolase deficiency could be readily detected. Usually, after combined reverse phase and cation exchange column chromatography, the effluent was monitored concurrently with spectometric and amperometric detection systems.

Bennett and Bradley (1984) used an Aminex HPX-87H (8% crosslinked, particle size 9 μm) cation exchange column, 300 x 7.0 mm, protected by a 5 cm guard column packed with the same resin. Acid separation at ambient temperature was achieved at a flow rate of 0.7 ml/min using 0.008 M sulfuric acid in water (pH 2), while acid detection was at 210 nm. An internal standard of 3-phenyl propionic acid was used since its retention time of 39 min was greater than the majority of the acids quantified.

Determination of organic acids by HPLC in diagnosis of acidemias in humans and various organic acid metabolic defects was reported by Tahara *et al.* (1984). Shodex Ionpak KC-811 anion exchange resin, 250 x 4.5 mm, was applied at 45°C. The eluent, 0.1% phosphoric acid, was run at a flow rate of 1 ml/min, and acid detection was at 210 nm. Lactic, pyruvic, methylmalonic and propionic acidemias could readily be detected in urine samples at a detection limit of 5 μg for each acid.

Buchanan and Thoene (1981) broadened the HPLC study of inborn acid metabolic disorders. In addition to carboxylic acids, they determined acids with functional groups, e.g. phenyl- (aromatic), hydroxyl, oxo (α-ketocarboxylic) or α,β-unsaturated organic acids. The separation column was again Aminex HPX-87 cationic exchange resin, while the eluent was 4.5 mM sulfuric acid at a flow rate of 0.8 ml/min at ambient temperature. The detection system consisted of a UV detector set at 200 nm and, in series upstream, a solid voltammetry detector, i.e. a mirror-polished, glassy carbon electrode set at +1.15 V vs an Ag/AgCl reference electrode. Phenolic, enenolic, oxalic and α-keto carboxylic acids were readily oxidized at the set potential and thus quantitated by a second linear recorder. The samples assayed were clarified through a 0.3 μm Millipore filter and injected into the column via a loop injector. Such a dual detection system permitted ready detection of inborn errors such as methylcitrate, oxalic, uric, orotic (urea cycle enzymopathies), pyruvic and branched chain ketoacidurea, along with phenylketonuria, tyrosinemia (p-hydoxyphenyllactic acid) and some other inborn errors.

The advantages of partially-quaternized dietylaminoethyl derivative of DEAE (TM DEAE-spheron) over the microporous polystyrene anion resins for anion exchange chromatography of organic acids were demonstrated by Vratny *et al.* (1983). Such columns, used at 60°C with 0.6 M sodium sulfate as mobile phase, were suitable for the separation of organic acids found mostly in agricultural and food samples of plant

origin (α-ketoglutaric, citric, malonic, oxalic, succinic, tartaric, malic, acetic, lactic, glycolic and pyrrolidone-carboxylic acids).

The partially-quaternized diethylaminoethyl derivative was synthesized from hydroxyethyl methaacrylate copolymer, the latter being supplied commercially as spherical micro-particles (TM Spheron-40, average particle size 14.3 μm). The fully-quaternized, strongly basic anion exchanger was commercially available and had a polystyrene matrix with a mean particle size of 10.2 μm. The latter was used as a reference column.

The common ion exchange chromatography, anion exchange and ion exclusion chromatography on cation exchangers have the shortcoming of slow diffusion within their swollen polymer. Vratny *et al.* (1983) found that, by using semi-rigid macroporous ion exchangers based on 2-hydroxyethyl methacrylate copolymers of DEAE, the shortcomings of the classical swelling microreticular ion exchangers (the volume of which strongly depends on the nature of the mobile-phase and its flow rate in the column) were eliminated. Thus, the anion exchanger based on hydroxyethyl methacrylate copolymer was better suited for rapid chromatographic separation of organic acids. The authors concluded that, on such columns with sodium sulfate as mobile phase, adsorption interactions occur with di- and tricarboxylic acids, but much less so with monocarboxylic acids.

The retention of organic acids was significantly affected by column temperature. A temperature increase from

30 to 60°C resulted in an increase in column efficiency but also led to an inversion in column selectivity for acid pairs α-ketoglutaric-itaconic and oxalic-citric. Nevertheless, the major advantage of the method applied by Vratny *et al.* (1983) was the quality of organic acid separation and its independence from the polarity of the injected sample (in the reverse phase method on silica, C_{18} sample dissolved in ethanol instead of water brings about a change in elution sequence). Also, as stated by the authors, in contrast to ion exlusion cation exchange columns, the anion exchange columns do not need a removal of interfering cations. Lastly, the authors suggested for routine analytical application a column packing of DEA-s spheron in sulfate ionic form, 250 x 6 mm, to be used at 60°C with an eluent flow rate of 1 ml/min, and, for a total of 20 organic acids, UV detection at 210-220 nm.

Neutral resins or propylamine columns have also been applied in organic acid separation by LC. Shaw and Wilson (1983) used such columns to separate malic, citric and succinic acids from some citrus fruits and cherry juices. Prior to analysis, all juice samples were clarified by centrifugation at 12,000 x g and then subjected to a preliminary separation on an ion exchange column in order to remove interfering compounds. The samples were first passed through a cation exchange resin (Bio-Rad AG-MP-50(H^+), 50-100 mesh), then through an anion exchange resin (Bio-Rad AG-MP-1(formate-), 50-100 mesh). The latter column was required for removal of sugars and other neutral compounds.

The acids were then washed out with 9 N formic acid, followed by water. The formic acid solution was evaporated to dryness, as was the water wash. The residues were redissolved in small volumes of water, combined, filtered through 1.2 and 0.22 µm Millipore filters, then injected into the LC. A DuPont Zorbax-NH_2 (propylamine) column was used with 0.075 M NaH_2PO_4 at a pH of 4.4 as eluent at a flow rate of 1.1 ml/min. Detection of the acids in the effluent was performed with a UV detector set at 206 nm.

In the past ten years reports have claimed that consumption of fermented or nonfermented dairy products lowers the content of human serum cholesterol (Mann and Spoerry, 1974; Hepner *et al.*, 1979). It was suggested that dairy products contain a "milk factor" which decreases cholesterol level. Ward *et al.* (1982) identified uric acid as the inhibitor of cholesterolgenesis in human milk. Uracil and orotic acids were designated as inhibitors of cholesterol synthesis in cow's milk by Papa *et al.* (1980). In addition, 3-hydroxy-3-methylglutarate (HMG) was suggested as another possible "milk factor" (Mann and Spoerry, 1974). These suggestions provided impetus for research endeavours to follow possible changes in the contents of orotic and uric acids and HMG in milk subjected to fermentation. Yogurt was the product studied in detail.

Haggerty *et al.* (1984) produced yogurts by using different strains of *Lactobacillus bulgaricus* (three patented strains, 201, 202 and 203) and *Streptococcus thermophilus* (two patented strains, EB6 and MC) and then

analyzed the levels of orotic, uric and HMG acids by using liquid chromatography.

The authors prepared the yogurt extracts by using 95% ethanol as a solvent, containing 1 volume percent of 5% oxalic acid. The samples were agitated for 1 min then centrifuged for 5 min at 7,000 x g. The clear supernatant was filtered through Whatman No. 4 paper, then evaporated to dryness. The residue was redissolved in distilled water, and filtered through Whatman No. 4 paper and then through a 0.45 μm Millipore filter.

Separation of the acids was achieved on an Aminex HPX-87 ion exclusion column (300 x 7.9 mm) using 0.003 N sulfuric acid as a mobile phase at a flow rate of 0.6 ml/min.

All yogurt samples (with an average total milk solids of 3.5%) showed a decrease in orotic acid of 15-53% after fermentation (as compared to fresh skim milk or nonfat dried milk solids), and no change in uric acid levels. However, eluted HMG, though it had a similar UV spectrum and coeluted with chemically-pure HMG, had different R_f values when assayed by TL or paper chromatography. Thus, the authors concluded that true HMG is not present in milk or yogurt. The coeluent level was reduced in six and was increased in two yogurt sample fermented by given strains of micro-organisms. Of interest was the orotic acid concentration reported by the authors. In eight yogurt samples assayed, the orotic acid ranged from 17.3 to 32.4 ppm. This was less than 72-83 ppm found in fresh fluid milk or 32-72 ppm in

yogurt as reported by Marsili *et al.* (1981). Also, the concentration of uric acid found in this study was much less, at 14.2-18.2 ppm, than the 31 ppm reported by Marsili *et al.* (1981). Based on these results, Haggerty *et al.* (1984) concluded that in yogurt production strain-induced differences do not exist in respect to levels of orotic and uric acids.

Jager and Tschager (1983) provided some organic acid data for Emmental cheeses. The extract obtained for LC was prepared from 5 g cheese treated with 60-70 ml water and readjusting the water phase pH to 7.0-7.5 ml with 1 N NaOH. This mixture was heated in a boiling water bath for 30 min, then cooled to 20°C, made up to 100 ml, and filtered through a fluted paper. The aqueous extract was further purified through an anion exchange column AG-X8(Cl^-), 200-400 mesh. A 5 ml aliquot was applied to the column and the column was washed with water to remove interfering compounds. Elution was achieved using a salt gradient. The initial eluent, 8 ml of 0.1 N NaCl, eluted pyruvic, lactic, formic, acetic, propionic and butyric acids. The next gradient, 5 ml of 0.2 N NaCl, eluted additional amounts of pyruvic, malic and succinic acids. This was followed by elution using the same volume and concentration of salt, giving a third fraction which consisted of citric and fumaric acids. Each of the three fractions was injected into an Aminex HPX-87 column, 300 x 7.8 mm. The first and second fractions were separated using 0.01 N H_2SO_4 as eluent at 15°C, while the third fraction was separated with 0.03 N sulfuric acid as eluent

at 18°C using a flow rate of 0.8 ml/min and a UV-detection system set at 210 nm.

Emmental cheeses studied by Jager and Tschager (1983) differed in their textural characteristics. Cheese A samples were taken from the production line and were classified as grade I. Cheese B samples had short and firm texture, while that of cheese C samples was weak and extensible. The textures of cheese A and B samples were readily distinguished by scanning electron microscopy. Sample A had clearly-defined large holes, while sample B had irregular holes separated by a porous matrix. These cheeses gave the following organic acid profiles: all contained pyruvic, lactic, formic, acetic, propionic, succinic and citric acids and were devoid of butyric and fumaric acids. The grade I cheese was distinguished by a higher content of lactic and lower content of propionic acid than samples B and C. Other organic acid profiles were practically the same. Butyric acid (content about 0.003%) was not detectable by this method. The difficulty in detecting such low levels was expected since organic acids with a keto functional group, or with a double bond, e.g. fumaric acid, have higher molar absorbances than acids with only a carboxyl group, e.g. butyric acid. No fumaric acid was detected in any of the Emmental cheeses.

By applying a modification of the method of Turkelson and Richards (1978), Marsili *et al.* (1981) used liquid chromatography for determination of organic acids in dairy products. Simultaneous and typical analytical results were

reported for the first time for orotic, citric, pyruvic, lactic, uric, formic, acetic, propionic, butyric and hippuric acids in whole milk, culture buttermilk, sour cream, cottage cheese, yogurt, cheddar cheese, blue cheese and skim milk powders.

The authors used a Varian model 5020 LC equipped with a manual 10 µl loop injector and a variable wavelength detector set at 220 and 275 nm, and quantitation was based on measuring only peak heights. The column used was a 300 x 7.8 mm i.d. Aminex HPX-87, an 8% crosslinked strong cation exchange resin with a particle size of 0.9 µm. Separation of acids was achieved by ion exclusion and partition chromatography with an isocratic mobile phase of 0.009 N sulfuric acid at a flow rate of 0.7 ml/min and a temperature of 65°C. HPLC grade acetonitrile was applied for sample preparation as a solvent and as a protein-denaturing agent. Analyzing samples at 220 and 275 nm, the authors quantitated formic and uric acids despite their coelution. Recovery was over 90% for all acids except butyric, for which the average recovery was 85.6%.

In the above study, typical chromatographic runs revealed the presence of formic acid in buttermilk, cheddar and blue cheeses, while the content of other acids was generally inversely related to the dry matter content of the dairy product. Lactic acid levels in fermented products were a maximum of 14,550±150 µg/g for plain yogurt, followed by sour cream at 8,410±160 µg/g and lower levels in cultured buttermilk and sharp Cheddar cheese, averaging 5,140 and

5,890 µg/g, respectively.

3. EXPERIMENTAL

3.1 Materials

3.1.1 Samples of dairy products

Samples obtained from the Northern Alberta Dairy Pool (NADP), Edmonton, included one of each of the following finished products: raw milk, skim milk, partly skimmed milk (2% butterfat milk), homogenized milk, cereal cream and whipping cream. Samples were obtained at each stage of the production process for each of the following products: cultured buttermilk, natural yogurt, sour cream and cottage cheese. These included samples before inoculation, during and after fermentation, and the finished product.

Samples obtained from the NADP, Barrhead, included: raw milk, buttermilk, skim milk, buttermilk powder and skim milk powder.

The following samples were taken from the Glenwoods Cheese Plant, Glenwoods, Alta.: white Cheddar at each stage of the production process and through six months of ripening.

From Neapolis Dairy Products Ltd., Didsbury, Alta., the following cheeses were sampled at each stage of their respective production processes: Camembert (also sampled throughout six weeks of ripening) and Quark cheese.

From the Alberta Cheese Company, Calgary: Pressed Mozzarella at each stage of the production process and at four weeks of ripening; Ricotta cheese (whey cheese) at each

stage of the production process; and stretched Mozzarella (pizza cheese) at each stage of the production process and through two weeks of ripening.

A number of imported cheeses (finished product only) were obtained on the Alberta market (major department stores in Edmonton). From Denmark: Camembert, Danablue, Feta, Havarti and Samsoe. From France: Brie, Camembert, Marcillat (goat's milk cheese), Port Salut and Roquefort (ewe's milk cheese). From the Netherlands: Edam and Gouda. From Switzerland: Appenzell, Emmental and Gruyère.

3.1.2 Chemicals

Sulfuric acid, reagent grade (assay 95.5–96.5%; 36.0214N), was obtained from Fisher Scientific Co., Fair Lawn, NJ.

Acetonitrile, HPLC grade (UV cutoff at 190 nm), was obtained from Caledon Laboratories Ltd., Georgetown, Ont.

The following analytical grade organic acids were used as standards:

- *Acetic acid*, Caledon Laboratories Ltd., Georgetown, Ont.
- *Butyric acid*, Fisher Scientific Co., Fair Lawn, NJ.
- *Citric acid* , Baker Chemical Co., Phillipsburg, NJ.
- *Formic acid*, BDH Lab Chemicals Division, Toronto, Ont.
- *Hippuric acid* (sodium salt hydrate), Aldrich Chemical Co., St. Paul, WI.
- *Lactic acid*, Serva-Feinbiochemica, Heidelberg, W.

Germany.

- *Orotic acid* (monohydrate), Sigma Chemical Co., St. Louis, MO.
- *Propionic acid*, Fisher Scientific Co., Fair Lawn, NJ.
- *Pyruvic acid* (sodium pyruvate), Sigma Chemical Co., St. Louis, MO.
- *Succinic acid*, Sigma Chemical Co., St. Louis, MO.
- *Uric acid*, Fisher Scientific Co., Fair Lawn, NJ.

No acidic impurities were detected during the analysis. Standard solutions were prepared in Milli-Q water (Millipore Ltd., Mississauga, Ont.).

3.2 Equipment

The liquid chromatographic system was obtained from Bio-Rad Laboratories Ltd., Richmond, CA. It consisted of the following parts:

The *column* (model 1321) was an Aminex HPX-87H column (300 x 78 mm) packed with an 8% crosslinked strong cation exchange resin in the hydrogen form with 9 μm diameter. Organic acids are separated by the mechanism of ion exclusion.

The *Micro-Guard HPLC Column Protection System* consisted of a cartridge (40 x 4.6 mm) packed with Aminex HPX-85H resin for ion exclusion (in organic acid analysis). It was placed between the injector and the analytical column to guard the column from degradation due to particulate matter, irreversibly-bound material and aggressive components in

sample or solvent.

The *column heater* was applied for separation at elevated temperatures (40-70°C) to resolve closely-eluting peaks, and to prevent the undesired absorbance effect and variability in sample retention.

The *detector* (model 1305) was a variable wavelength ultraviolet detector for liquid chromatography. It consisted of an optical unit (including the flow cell) and a control unit. A standard deuterium lamp covers wavelengths from 190 to 350 nm, while an optional tungsten lamp expands the wavelength range to 600 nm.

The *pump* (model 1330) was a dual piston constant flow, low pulsation solvent delivery system. The delivery flow rate ranges from 0 to 9.9 ml/min.

The *sample injector* and injector brackets (model 7125) were manufactured by Rheodyne Inc., Cotati, CA. The injector was a rotary six-port valve with external sample loop (20 µl) which operates at pressures up to 7000 psi. Loading of the sample loop was accomplished with a syringe through a needle port in the valve.

Injections were made with a 50 µl low pressure, gas-tight *volumetric syringe* with removable needles, supplied by Hamilton Co., Reno, NV.

Teflon and type 316 stainless steel tubings and surfaces were used throughout the system to prevent damage to components from the acidic buffers.

The *integrator* (model 3388), obtained from Hewlett Packard (Avondale, PA), was used to quantitate the peaks.

The refrigerated general purpose laboratory centrifuge (model J-21B) with fixed angle rotor (model JA-20) was obtained from Beckman, Palo Alto, CA.

3.3 Methods

3.3.1 Procedures in taking samples in the industry

All samples were collected by the author from different dairy plants in Alberta, under sanitary conditions required in the dairy industry. Samples were cooled immediately in ice water and kept refrigerated. Extraction was performed the same day and the extracts were kept refrigerated prior to HPLC analysis.

3.3.2 Analytical procedures

One solid sample of each cheese was analyzed. The samples were crushed with mortar and pestle to facilitate mixing with the solvent (acetonitrile and water, 4:1 v/v). Non-homogeneous samples of curd and whey were homogenized using a Lab-Blender 400 (Colwarth, Bury St. Edmunds, UK). Aqueous dilutions (20% w/w) were prepared for powdered samples.

5.00 g of dairy sample were homogenized with 5.0 ml of Milli-Q water. The mixture was then combined with 20 ml of acetonitrile in 50 ml polypropylene centrifuge tubes (Fisher Scientific Co., Fairlawn, NY). The mixture was shaken for 1 min in a vortex mixer and centrifuged at 7,000 rpm. Before injection into the column, the supernatant was filtered into

the column through 0.20 μm nylon filters with a stainless filter holder equipped with a stainless steel support screen (Rainin Instrument Co., Woburn, MA). Samples were injected with a 50 μl Hamilton syringe into the 20 μl sample loop.

Four aqueous dilutions of each standard organic acid were prepared for the calibration curves.

The working chromatographic conditions were as follows:

- eluent concentration: 0.02N and 0.0045N H_2SO_4
- flow rate of the mobile phase: 0.7 ml/min
- column temperature: 65°C
- sample size: 20 μl
- UV detection: 220 and 275 nm
- quantitation: based on peak area measurements

Cheeses were ripened in low temperature incubators (manufactured by Precision Scientific, Chicago, IL) at 6-8°C, under the same conditions as in the cheese plants.

3.4 Production of Dairy Products

This section describes the production processes of the various dairy products used in this study. Some details on processing and chemistry of these products are available from a number of sources (Alfa-Laval; Davis, 1976; Hall and Hendrick, 1971; Henderson, 1971; Kosikowski, 1966; Kosikowski and Mocquot, 1958; Lampert, 1975; Ling, 1948; Newlander, 1977; Rasic and Kurmann, 1978; Richardson, 1985; Scott, 1981; Simon, 1956; Webb and Johnson, 1956; Wilcox, 1971; and Wilster, 1959).

3.4.1 Fluid products, non-fermented

Raw milk was collected by NADP trucks from the bulk tanks of surrounding dairy farms. It was refrigerated from the time of milking until it reached the plant, where it was kept refrigerated in raw milk bulk tanks until used for processing (not more than two days). The average gross composition of cow's milk was: water, 87%; fat, 3.9%; lactose, 4.9%; proteins, 3.5%; and ash (minerals), 0.7%. Skim milk, partly skimmed milk (2% butterfat milk) and homogenized milk, as well as cereal cream and whipping cream, were produced from raw milk (Table 3.1). Partly skimmed milk and homogenized milk were a mixture of skim milk, cream and vitamins A and D. Cereal cream, or Half and Half, was a mixture of milk and cream that contained a minimum of 10% butterfat (BF). Whipping cream was a mixture of milk, cream, and any one of several different stabilizers (guar gum, propylene glycol, alginate, carrageenan and locust bean gum) that contained a minimum of 30% BF. The products were treated in the following way:

- raw milk
- separation into skim milk and cream (centrifugal separator with clarifying and standardizing ability, supplied by Sentrico, Westin, Ont.)
- blending of skim milk and cream to form: partly skimmed (2% BF) milk, homogenized milk, cereal cream and whipping cream
- homogenization (high pressure homogenizer, purchased from Gajlin, Everati, MA)

Table 3.1. NADP product specifications.

Product	Butterfat (%)	Pasteurization		Homogenization Pressure (kg/cm^2)	Additional Ingredients
		Temp (°C)	Time (sec)		
Skim milk	0.01-0.02	78	16	35	---
Partly skimmed milk	1.98-2.05	78	16	110	vitamins A,D
Homogenized milk	3.23-3.26	78	16	110	vitamins A,D
Whipping cream	32.5	80	150	110	carrageenan
Cereal cream	10.0	80	150	---	---

- addition of ingredients (stabilizers supplied by Food Speciality, Holton Hills, Ont.; and vitamins from Kingsway Chocolate, Mississauga, Ont.)
- HTST (high temperature short time) pasteurization (continuous flow plate heat exchanger, from APV, Westin, Ont.)
- packaging of the product

3.4.2 Fermented products

3.4.2.1 Cultured buttermilk

Buttermilk was a partly skimmed, pasteurized milk that had been fermented by a lactic culture (*Streptococcus lactis*) and by aroma bacteria (*Leuconostoc cremoris*). The resulting acid curd was characterized by its pleasant aroma and flavour. Cultured buttermilk contained 1.8% BF and 88.5% moisture. It was made as follows:

- blending of ingredients (cream, skim milk, 2% of skim milk powder and 0.005% gelatin)
- homogenization (175 kg/cm^2; high pressure homogenizer)
- HTST pasteurization (80°C for 150 sec; continuous flow plate heat exchanger)
- addition of *Streptococcus lactis* and *Leuconostoc cremoris* as starters (supplied by Nordica Ltd., Mississauga, Ont.)
- setting at 23°C for 14 hr or until pH reaches 4.6
- agitation and cooling to 4-5°C
- packaging and storing at 4°C (final pH=4.6)

3.4.2.2 Natural yogurt

Yogurt, as a cultured milk product, was turned into a thick creamy curd by the action of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. It contained 2.0% BF and 86.3% moisture, and was produced as follows:

- blending of ingredients (cream, skim milk, 2% skim milk powder and 0.6% gelatin)
- homogenization (175 kg/cm²; high pressure homogenizer)
- pasteurization (80°C for 150 sec; continuous flow plate heat exchanger).
- addition of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (1:1) as starters (supplied by Nordica Ltd., Mississauga, Ont.)
- setting at 43°C for 4 hr (or until pH reaches 4.4)
- agitation and cooling to 4-5°C
- packaging and storing at 4°C (final pH=4.4)

3.4.2.3 Sour cream

Sour cream was an acid gel accompanied by delicate flavour which resulted from the growth and activity of *Streptococcus lactis* and flavour-producing *Leuconostoc cremoris* on light cream. It contained 15.5% BF and 74.5% moisture, and was produced as follows:

- blending of ingredients (cream, skim milk, 3% skim milk powder and 0.25% gelatin)
- homogenization (175 kg/cm²; high pressure homogenizer)
- HTST pasteurization (80°C for 150 sec; continuous

flow plate heat exchanger)

- addition of *Streptococcus lactis* and *Leuconostoc cremoris* as starters (supplied by Nordica Ltd., Mississauga, Ont.)
- setting at 23°C for 15 hours (or until pH reaches 4.6)
- agitation and cooling to 4-5°C
- packaging and storing at 4°C (final pH=4.6)

3.4.3 Dehydrated products

These involved skim milk and buttermilk in powdered forms. For their production, raw milk was separated (at 49°C; centrifugal separator purchased from De Laval, Peterborough, Ont.) into cream (38% BF) and skim milk (0.04% BF). The cream was used in the production of butter (80.5% BF and 17% moisture) by a continuous churning process, with buttermilk (0.9% BF) as a byproduct. Buttermilk powder contained 5.5% butterfat and 4.0% moisture, and skim milk powder contained 0.9% butterfat and 4.0% moisture. The buttermilk and skim milk were dehydrated as follows:

- liquid buttermilk or skim milk
- preheating to 80°C
- 1st stage evaporation (64°C and vacuum pressure = 98.3 kPa)
- 2nd stage evaporation (52°C and vacuum pressure = 81.3 kPa), concentrating the product to 45% solids (the falling film evaporator was obtained from Majorica Brothers, Chicago, IL)

- spray drying (product is atomized in a stream of hot air, temperature = 215°C; the spray dryer was obtained from Majorica Brothers, Chicago, IL)
- air lock (the secondary burner reheats the product to 99°C)
- transfer and mixing of product by auger
- 1st stage cyclon separator (air 99°C)
- 2nd stage cyclon separator (recovers small powder particles)
- cooling to 25°C
- packaging in polyethylene bags

3.4.4 Cottage cheese

Cottage cheese, soft unripened white cheese, was made from milk to which cream, salt, starters and rennet were added. The starters were *Streptococcus lactis* and *Leuconostoc cremoris*. It was a lactic acid precipitated type of cheese, to which the rennin enzyme was applied only for the purpose of speeding up curd precipitation. The product with 2.0% BF and 72.6% moisture was produced by the following process:

- skim milk (called cheese skim)
- HTST pasteurization (72°C for 16 sec; pasteurizer obtained from APV, Westin, Ont.)
- blending of ingredients (cheese skim: skim milk, 1.5% of skim milk powder, 0.01-0.02% $CaCl_2$ as mineral supplement)
- addition of *Streptococcus lactis* and *Leuconostoc*

cremoris as 4% starter culture (supplied by Nordica Ltd., Mississauga, Ont.) at 31.5°C

- addition of 0.00001% of natural rennet (single strength, supplied by Hansen's Laboratory Inc., Mississauga, Ont.); rennet activity is 88 units of rennin per ml, or enough to obtain desirable firmness of the cheese cloth in 5 hr
- incubation at 31.5°C for 4-6 hr (until pH reaches 4.7)
- cutting into curds at 38°C
- cooking the curd to the right firmness (until 49-54.5°C)
- draining of whey
- curd rinsing (3 times)
- addition of salt (NaCl, fine mesh, purchased from Sifto, Unity, Sask.)
- addition of cheese dressing (0.17%), made by blending milk and any of the following stabilizers: guar gum, mono and diglycerides, xanthan gum, locust bean gum or polysorbate 80 (which was then homogenized at pressure = 175 kg/cm^2, and pasteurized at 85°C for 150 sec)

3.4.5 Cheddar cheese

Cheddar, a very close-textured firm-bodied cheese with a pale creamy colour and a characteristic flavour, was obtained with the activity of *Streptococcus cremoris* (a mixture of five strains, the exact composition of which was

changed every 2-3 weeks). After packaging, it contained 34.0% BF and 36.5% moisture. It was produced by the following process:

- whole milk (3.6% BF)
- HTST pasteurization of milk (72°C for 15 sec; pasteurizer obtained from Crepaco, Westin, Ont.)
- addition of 2% starter culture of *Streptococcus cremoris* (obtained from Bio Lac Company, Logan, UT), at 30°C
- ripening for 10 min (until titrable acidity reaches 0.22% lactic acid)
- addition of Annato colouring (0.003%, obtained from Jenco Machinery Ltd., Calgary, Alta.)
- addition of 0.3% rennet (microbial and natural rennet, 2:1), to obtain the desired firmness of the cheese cloth in 25 min; obtained from Milles Laboratories, Beossarb, Ont.
- curd setting for 25-30 min
- curd cutting
- after 15 min, curd cooking (38°C) for 30 min
- whey draining (pH=6.1)
- cheddaring and curd piling
- end of cheddaring (2.5 hr later; acidity 0.51-0.6%)
- curd milling at 33°C
- dry salting (0.2% NaCl)
- 25 min later, moulding (32°C)
- pressing (vertical press purchased from Falco, Montreal, P.Q.) overnight: P=295 kPa.

- vacuum packaging in polyethylene bags
- ripening at 8°C for 6 months

3.4.6 Camembert

Camembert, a soft surface mould (*Penicillium camemberti*) ripened cheese, was made by a starter culture consisting of both *Streptococcus lactis* and *S. cremoris*. The finished cheese contained 22% butterfat and 55% moisture. It was produced as follows:

- whole milk (3.5% BF)
- HTST pasteurization (75°C for 10 sec; pasteurizer purchased from APV, Westin, Ont.)
- addition of 2% culture consisting of *Streptococcus lactis* and *S. cremoris* (Flora Danica, supplied by Horan-Lally Co. Ltd., Mississauga, Ont.)
- after 20 min, addition of single-strength natural rennet to obtain the desired firmness of the cheese cloth in 35 min (0.012%; supplied by Hansen's Laboratory Inc., Mississauga, Ont.)
- addition of mould (0.005%), supplied by Laboratorium Dr. Kohler, GroB Lobke, W. Germany)
- cutting after 35 min (pH=6.22)
- moulding into forms
- turning 3 times per day (24-29°C)
- next day, salting in a brine bath (20% salt) for 35 min
- drying for 2-3 days at 20°C, RH=40%
- resting in the cure room for 7-8 days, at 23°C and

RH=50-60%

- drying in cold storage for 4 hr, at 2°C and RH=30%
- wrapping in perforated aluminum foil
- ripening for 4 weeks at 10°C

3.4.7 Quark

Butter culture (*Streptococcus cremoris*, *S. lactis* and *Leuconostoc cremoris*) is used for Quark production. Hence, Quark was essentially a lactic curd cheese, which contained 13.2% BF and 75.0% moisture. It was produced by the following process:

- whole milk (3.5% BF)
- HTST pasteurization (75°C for 10 sec; pasteurizer purchased from APV, Westin, Ont.)
- addition of culture consisting of *Streptococcus cremoris*, *S. lactis* and *Leuconostoc cremoris* (Flora Danica, supplied by Horan-Lally Co. Ltd., Mississauga, Ont.)
- addition 1 hr later of single-strength natural rennet (0.0007%) to obtain the desired firmness of the cheese cloth in 8 hr (supplied by Hansen's Laboratory Inc., Mississauga, Ont.)
- curd setting for 8 hr at ambient temperature
- slow curd pressing
- drainage of whey overnight, when pH=4.6
- addition of pasteurized chilled water (to soften the curd) and preservatives (0.02% potassium sorbate)
- packaging and storing at 4-5°C

3.4.8 Pressed Mozzarella

Mozzarella was produced from milk by the addition of a culture consisting of *Streptococcus lactis* and *S. cremoris*. The product contained 20% BF and 45% moisture. It was produced by the following process:

- partly skimmed milk (2.7% BF; separator from De Laval, Peterborough, Ont.)
- HTST pasteurization of milk (72°C for 16 sec; pasteurizer purchased from APV, Westin, Ont.)
- cooling to 32°C
- addition of 1.5-2.0% culture of *Streptococcus lactis* and *S. cremoris* (supplied by Hansen's Laboratory Inc., Mississiauga, Ont.)
- after 15 min, addition of single-strength natural rennet to obtain the desired firmness of the cheese cloth in 45 min (supplied by Hansen's Laboratory Inc., Mississauga, Ont.)
- curd setting for 45 min
- cutting (acidity=0.11%)
- after 15 min, agitation
- cooking at 38°C for 40-45 min
- drainage of whey (acidity=0.13%)
- washing with water at 21°C (acidity of whey=0.16%)
- drainage of 75% of the water
- presalting and stirring of the curd (NaCl, fine mesh, purchased from Sifto, Unity, Sask.)
- draining of whey (acidity=0.18-0.20%)
- curd salting (NaCl, fine mesh)

- agitation for 5 min
- hooping and pressing overnight (vertical press purchased from Falco, Montreal, P. Q.)
- vacuum packaging in polyethylene bags
- ripening at 6-7°C for 2-4 weeks

3.4.9 Ricotta

Ricotta cheese is produced by heating of acidified whey to 65°C. It is a high moisture, low fat, unripened cheese, with no culture or rennet added. Usually, pressed mozzarella whey was used in the production of Ricotta. It contained 0.5% BF and 75% moisture. It was produced by the following process:

- mozzarella whey (68% of total batch blend)
- heating to 65°C
- addition of 0.2% (of total batch blend) salt (NaCl)
- addition of 1% (of total batch blend) cream (30-35% BF)
- addition of 25% (of total batch blend) milk (3.3% BF)
- heating to 83°C
- addition of ricotta whey (6% of total batch blend), acidified with citric acid
- setting for 5 min, during which time curd rises to the top
- skimming of the curd
- curd draining into perforated plastic containers
- packaging, cooling to 4°C overnight and storing

3.4.10 Stretched or Pizza Mozzarella

Pizza cheese was a rennet curd cheese, where the curd was cooked at higher temperatures and acid ripened by a mixture of *Streptococcus lactis*, *S. cremoris*, *Lactobacillus bulgaricus* and *S. thermophilus*. When made from partly skimmed milk, it contained 15% butterfat and 52% moisture. It was produced by the following process:

- partly skimmed milk (2.3% BF)
- HTST pasteurization (72°C for 16 sec; pasteurizer purchased from APV, Westin, Ont.)
- cooling to 32°C
- addition of 1.5% culture (*Streptococcus lactis*, *S. cremoris*, *Lactobacillus bulgaricus* and *S. thermophilus*), supplied by Hansen's Laboratory Inc., Mississauga, Ont.
- setting at 30°C for 30 min
- addition of single strength natural rennet to obtain the desired firmness of the cheese cloth in 30 min (supplied by Hansen's Laboratory Inc., Mississauga, Ont.)
- after 30 min, curd cutting
- after 10 min, curd cooking (40°C) for 10 min
- after 10 min, draining of whey (pH=6.1)
- curd cutting, turning and piling; and whey is removed completely (pH=5.2)
- curd milling at 39°C (to form long strips)
- curd cooking in hot water (73°C) and simultaneous curd stretching

- curd moulding at 45°C
- curd cooling by dipping in cold water
- salting the cheese overnight in a 20% brine bath (5°C)
- vacuum packaging in polyethylene bags
- ripening for 2 weeks at 6-7°C

3.4.11 Danish cheeses

Although a country with a historically famous dairy industry, Denmark has never become known as an "original cheese" country. Many cheeses are produced, but the biggest export cheeses are: Camembert, Danish blue (Danablue), Havarti, and Samsoe.

3.4.11.1 Camembert

Camembert is a soft ripened cheese, made from pasteurized milk by a procedure similar to that of French Camembert (see 3.4.12.2). Lactic acid bacteria are used as starters. *Penicillium camemberti* produces a white crust with a soft centre when ripened. It contains 50% BF in dry matter.

3.4.11.2 Danablue

Danish blue cheese is an imitation of Roquefort, but is made from cow's milk. It has only a limited ripening, and is lacking in the finer points of the traditional blue-veined varieties. It contains maximum 47% moisture and 60% BF in dry matter. It is manufactured in the same way as Roquefort (see 3.4.12.5), with the following exceptions:

- homogenized cow's milk is used
- ripening period is shorter
- salt content is higher
- mould spores (*Penicillium candidum* and *P. roqueforti*) are incorporated into the curd, and stabbing or piercing is very effective in promoting their growth

3.4.11.3 Feta

Although usually made from sheep's milk, cow's milk has been used in the production of Feta cheese. It is a soft, brine-pickled cheese which contains 22% BF and 52% moisture. It is produced in the following way:

- pasteurization of cow's milk (65°C, 30 min)
- cooling to 32°C
- addition of 1-3% starter (*Streptococcus lactic* and *S. cremoris*)
- addition of 0.02% $CaCl_2$
- addition of rennet (0.025%)
- after 50-150 min, cutting of the curd
- after 10-15 min, drainage of whey
- dry salting for 2 days (6% salt incorporated)
- immersion in 16% brine for 24 hr
- ripening on a drainer for 8-15 days

3.4.11.4 Havarti

Havarti is a semi-hard variety that is softer than other semi-hard cheeses due to the growth of microorganisms on the rind. It has a yellow body with small, irregularly-

shaped holes. It contains maximum 50% moisture and 45% BF in dry matter, and is produced in the following way:

- pasteurization of milk (3% BF) at 70-72°C for 15 sec
- cooling to 30°C
- addition of 0.7% starter, saltpetre (0.01%) and rennet (0.03%)
- after 35 min, cutting
- stirring for 15 min
- drainage of 33% of whey
- addition of hot water (55°C) to reach 36°C
- addition of salt (0.2%)
- stirring for 10 min
- drainage of remaining whey
- moulding
- frequent turning
- immersion in water (18°C) overnight
- next day, immersion in 24% brine (12°C) for 2 days
- drainage for 1 day (14°C)
- ripening for 5 weeks (16°C and 90% RH)
- ripening for 1 week (11-12°C and 80% RH)
- packaging

3.4.11.5 Samsoe

Samsoe is a Swiss-type cheese that contains 44-52% moisture, and 45% BF in dry matter. It has a firm body and holes, similar to Swiss cheeses. Samsoe produced for export is matured for 5 months. It is produced in the following way:

- pasteurization of milk (3% BF) at 70-72°C for 15 sec

- cooling to 30°C
- addition of 0.7% starter
- after 10 min, addition of rennet (0.03%)
- after 30 min, cutting
- stirring for 30 min
- drainage of 33% of whey and stirring of curd for 5 min
- addition of hot water (65°C), to reach 37°C
- stirring for 20 min
- addition of salt (0.02%)
- stirring for 50 min
- drainage of whey
- moulding
- pressing for 25 min in vat
- pressing outside vat (20°C), for 30 min without cloth
- wrapping in cloth and turning
- pressing for 3 hr (20°C)
- pressing for 16 hr (14°C)
- immersion in 24% brine (12°C) for 3 days
- draining for a day at 14°C
- after 7 days, transfer of cheese to ripening room (17°C and 85% RH) for 4 weeks
- transfer to second ripening room (11-12°C and 80% RH) for 1 week
- transfer to third ripening room (10-11°C and 75-80% RH)
- waxing when cheeses are 6-7 weeks old

3.4.12 French cheeses

France leads all other nations in number of named varieties of cheeses and in cheese consumption.

3.4.12.1 Brie

Brie is basically the same as Camembert, a surface-mould-ripened soft cheese. It is one of the most delicate cheeses and the greatest care is necessary in its manufacture, ripening, storage and distribution. It contains maximum 56% moisture and minimum 40% BF in dry matter. Brie is produced in the following way:

- pasteurization of milk (65°C, 30 min)
- addition of lactic acid starter, rennet and moulds
- increase in temperature to 31°C
- after 2-3 hr, cutting with a wire knife
- hooping on a draining board
- draining at 18°C
- piling of hoops and standing for 24 hr
- hoops fastened with metal ring and standing for 1 day
- dry salting daily for next 2-3 days
- first stage of ripening in a well-ventilated room at 14°C
- after 8 days, developing of white mould (*Penicillium candidum*)
- cheese transferred to a chamber at 11°C and 85% RH
- under the influence of the mould growth, creamy-yellow curd changes to yellow and finally reddish

3.4.12.2 Camembert

Along with Brie, Camembert is one of the two most famous names concerned with soft, surface-mould-ripened flat cheese. It is fit for eating in 4 to 6 weeks. It contains maximum 48% moisture and minimum 45% BF in dry matter. It is produced in the following way:

- pasteurization of milk (65°C, 30 min)
- cooling to 33-34°C
- addition of 2% lactic acid starter
- 1 hr later, addition of rennet (0.016%)
- coagulation occurs in 25 min
- cutting in 70 min
- moulding on draining mats
- draining for 5.5-6 hr (acidity of whey, 0.6-0.7%)
- turning of cheese and standing overnight (room temp., 22°C)
- next morning, removal of moulds
- transfer to salting room (18-20°C)
- after 1-1.5 hr, dry salting and spraying with mould culture
- 3 hr later, turning and repeating the previous step
- 1 hr later, salting is repeated on the other surface
- turning of the cheese
- removal of mat and turning of the cheese
- transfer to another room (12-14°C), and storage for 10-12 days
- packaging
- soft lactic acid curd becomes firmer as it dries out

- mould develops on the coat
- coat changes from snow white to a slightly greyish creamy colour, and the body becomes softer and more velvety or waxy
- central white "chalky" layer steadily becomes thinner as the proteolytic and lipolytic enzymes produced by the *Penicillium candidum* (*camemberti*) diffuses inwards
- when chalky layer disappears, cheese is ripened

3.4.12.3 Marcillat

Marcillat is a goat's milk cheese, made following the basic Camembert procedure (see 3.4.12.2). It is a surface-mould-ripened cheese (*Penicillium candidum*). Lactic acid culture and salt are added. It contains 50% BF in dry matter.

Goat's milk is about as rich as or slightly richer than cow's milk. The flavour is usually strong and characteristic because of the predominance of low fatty acids (C_6, C_8 and C_{10}) in the fat of goat's milk.

3.4.12.4 Port Salut

Port Salut is the most famous of the French semi-hard varieties. It is elastic and the minimum fat in dry matter is 42%. The production process is as follows:

- pasteurization of milk (79°C, 10 sec)
- cooling to 34°C
- addition of 2% lactic acid starter (from Flora Danica)

- addition of 0.01% $CaCl_2$, colouring agent and rennet (0.022%)
- after 35 min, cutting of the coagulum
- drainage of whey and stirring for 35 min (until acidity of whey reaches 0.11%
- drainage of more whey and curd heating to 36-40°C
- moulding on draining boards
- pressing of the moulds for a few hours and regular turning
- salting of cheese and immersing in brine for 7-12 hr
- ripening in cellars at 12°C and 85% RH

3.4.12.5 Roquefort

Roquefort is the most famous ewe's milk cheese and one of the most famous blue-veined varieties in the world. It is matured in natural caves in Roquefort. It is very microbiologically stable and contains 52% moisture and 48% BF in dry matter.

Ewe's milk is richer in fat, non-fat solids, and certain vitamins than cow's milk, and the higher content of certain lower fatty acids results in the production of a characteristic and strongly flavoured cheese. Roquefort is produced as follows:

- sheep's milk, not pasteurized or skimmed (28-30°C)
- addition of rennet
- after 2 hr, cutting of coagulum
- drainage of whey
- moulding in perforated cylindrical moulds and simultaneous sprinkling of *Penicillium roqueforti*

spores

- draining for 3-5 days on tables (piled 2 or 3 moulds high on the 2nd or 3rd day)
- chilling at 12°C and 90% RH
- after 5 days, salting by hand over a 3-day period
- stabbing of cheese, which results in an atmosphere of 2-7% oxygen in the interior of the cheese
- maturing in caves at 5-10°C and 95% RH
- scraping and brushing every 14 days for the 3 months' ripening in the caves

3.4.13 Cheeses of the Netherlands

The two best known Dutch varieties are Edam and Gouda. Holland is considered to be one of the most progressive cheesemaking countries in the world (Davis, 1976). Edam and Gouda were investigated in this study.

3.4.13.1 Edam

Edam is also known as "Dutch" or simply "red cheese." It has a close texture and milk flavour, with 38-44% moisture, and 40-45% BF in dry matter. It is produced in the following way:

- pasteurization (75°C, 15 sec) of milk (2.45-2.95% BF)
- addition of $CaCl_2$ (up to 0.02%)
- addition of $NaNO_3$ (up to 0.02%)
- colouring
- addition of 0.5% starter (30°C) and rennet (0.03%)
- after 30 min of incubation, cutting

- stirring for 20-30 min
- draining of 50% of whey
- addition of water (up to 25%) at 50-60°C
- stirring for 30 min
- after 30 min, temperature adjusted to 36°C (curd pH=5.3)
- drainage of whey
- pressing of curd for 20 min
- moulding
- pressing for 30 min-3 hr
- dipping in hot whey (50-55°C) and trimming
- pressing at greater pressure for longer time (15-20°C)
- salting in 21% brine for 2 days
- drainage and drying at 12-15°C, RH=85-90%
- wrapping or waxing
- ripening at 15°C for 6 weeks

3.4.13.2 Gouda

Gouda is similar to Edam, but is not as firm and rubbery, and has a more waxy body, with a few small holes. It has a yellow colour, and a bland flavour. It has 51% BF in dry matter, and the maximum moisture at 10 days is 44-45%. The culture used is a mixture of *Streptococcus lactis*, *S. lactis var. hollandicus* and *S. diacetylactis*. Gouda is produced in the following way:

- pasteurization of whole milk (75°C, 15 sec)
- cooling to 31°C
- addition of starter (0.5%), $NaNO_3$ (0.005%) and $CaCl_2$

(up to 0.02%)

- addition of Annatto colouring (0.022%)
- incubation for 10-30 min
- addition of rennet (0.025%)
- after 25-30 min, cutting
- stirring for 20 min
- draining of 50% of whey
- addition of hot water (60°C), to reach 40°C
- after 30 min, pressing of the curd under the whey
- cutting into blocks
- removal from whey
- moulding
- pressing for up to 5 hr (acidity of whey, 0.3%)
- immersion in saturated brine (15°C) for 3-5 days (cheese pH=5.2)
- drying for 1 day
- packaging in foil or coating with plastic
- ripening at 15°C for 6 weeks

3.4.14 Swiss cheeses

"Swiss cheese" means a hard cheese with well developed holes or eyes. The two best known varieties are Gruyère and Emmental. The characteristic eyes of Swiss cheese are the result of secondary fermentation in the cheese (propionic acid formation). The high temperature employed for Gruyère is largely responsible for the fewer and smaller holes, compared with Emmental.

3.4.14.1 Appenzell

Appenzell resembles Emmental cheese (see 3.4.14.2), with a few exceptions. Appenzell has only a few small holes and contains 50% BF in dry matter. The culture used is a lactic acid starter and a propionic acid culture. During production, the cheese is immersed for a few days in spiced white wine or cider. In the ripening stage, the cheese is rubbed with brine-soaked cloths at intervals.

3.4.14.2 Emmental

Emmental is the most important variety in Switzerland. It has many large holes, and contains 45% BF in dry matter. It is produced in the following way:

- previous evening's milk kept cool overnight and inoculated with 0.01% starter (*Streptococcus lactis* and *S. cremoris*)
- morning milk added
- standardization to 2.8-3.1% BF
- inoculation with *Streptococcus thermophilus* and culture of *Lactobacillus helveticus*, as well as propionic acid bacteria
- increase of temperature to 31-32°C
- addition of rennet (0.016%) to clot milk in 20-30 min
- addition of pure water to the rennet
- cutting with a wire knife
- stirring
- increase of temperature by 1 C° every 2 min, until 45°C

- increase of temperature by 1 C° per min, until 53-57°C
- stirring terminated 45 min after start of cooking
- addition of a few litres of cold water
- collection of curd in cloth
- moulding of clothed cheese and pressure applied (up to 15 kg/1 kg of cheese)
- turning of cheese regularly for 2 days
- removal of cloth
- dry salting in the mould for a day or two (10°C)
- immersion in brine (salt concentration high enough that cheese floats)
- turning once per day and sprinkling salt over the exposed surface
- after 1-2 days, removal from brine and transfer to a chilled room (10-12°C and 80-85% RH) for 8-10 days, where cheese is brushed, dry-salted and turned daily
- transfer to warm room (18-20°C and 80-85% RH) for 4-8 weeks, where cheese is regularly washed with brine and turned

3.4.14.3 Gruyère

Compared to Emmental, Gruyère is a drier cheese, with a more mature and sharper flavour. It has a waxy body and only a few small eyes, and contains 47-49% BF in dry matter. The method of production is the same as that of Emmental (see 3.4.14.2), with the following exceptions:

- milk is not standardized
- more starter added (0.5-0.7% *Streptococcus*

thermophilus and *Lactobacillus helveticus*)

- curd cut in bigger pieces
- maximum scald temperature 2 C° higher (up to 55-57°C)
- higher degree of pressing
- more salt worked into the cheese
- ripening temperature lower (12-18°C) and relative humidity higher (85-90%)
- during ripening, rind is rubbed with brine and *Bacillus linens* culture
- total manufacturing time: 8-12 months

4. RESULTS AND DISCUSSION

4.1 Assessment of the Applied LC Method

Organic acids were separated by liquid chromatography (LC) by the mechanism of ion exclusion chromatography, hence the acids emerged from the analytical column in an order closely following their pK values (Table 4.1) with 0.009 N sulfuric acid used as the mobile phase. In this case the elution sequence was as follows: orotic shouldered and often coeluted with citric acid; pyruvic, succinic, lactic, uric and formic acids as a single peak, followed by acetic, propionic and butyric acid peaks. The last acid eluted was hippuric acid. Even though its pK is 3.80, it was eluted last probably because of steric hindrance within the cross-linked resin(Table 4.2).

In order to improve the separation of orotic from citric acid, the mobile phase normality was increased to 0.02 N (pH 1.97). This normality provided satisfactory resolution of citric and orotic acids without altering the sequence of elution of other acids nor their retention times, except for hippuric acid for which the retention time increased from 31 to 33 min.

A further increase in normality of the mobile phase from 0.02 to 0.05 N sulfuric acid resulted in additional improvement in resolution of citric and orotic acids but caused lactic acid to coelute with uric and formic acids. There was a slight decrease in retention times of acetic and hippuric acids, while the retention times of propionic and

Table 4.1. Dissociation constants of some organic acids found in dairy products.

Acid	Temperature (°C)	Step	Aqueous solution[1] K	pK
Citric	20	1	7.10×10^{-4}	3.14
	20	2	1.68×10^{-5}	4.77
	20	3	6.40×10^{-7}	6.39
Orotic	20	1	3.98×10^{-3}	2.4
	20	2	3.16×10^{-10}	9.5
	20	3	---	>13
Pyruvic	25		3.2×10^{-3}	2.49
Succinic	25	1	6.89×10^{-5}	4.16
	25	2	2.47×10^{-6}	5.61
Lactic	25		1.38×10^{-4}	3.86
Uric	12		1.3×10^{-4}	3.89
Formic	20		1.77×10^{-4}	3.75
Acetic	25		1.76×10^{-5}	4.75
Propionic	25		1.34×10^{-5}	4.87
Butyric	20		1.54×10^{-5}	4.81
Hippuric	25		1.57×10^{-4}	3.80

Sources: Weast, R.C. (ed.). 1983. Handbook of Chemistry and Physics, CRC Press Inc., Boca Raton, FL; and Sober, H.A. (ed.). 1970. Handbook of Biochemistry, The Chemical Rubber Co., Cleveland, OH.

[1] $K = [H^+][A^-]/[HA]$ $\quad HA \rightleftharpoons [H^+][A^-]$ $\quad pK = -\log K$

Table 4.2. The effect of eluent normality on retention times (Rt, min) of organic acids separated by liquid chromatography as applied in this study.

Organic acid	Eluent (H_2SO_4)		
	0.009 N	0.02 N	0.05 N
Citric	6.80[1]	7.00	7.11
Orotic	6.80	7.62	8.85
Pyruvic	7.92	8.78	9.53
Succinic	9.92	10.16	9.91
Lactic	11.33	11.33	11.61
Uric	12.01	12.00	11.61
Formic	12.01	12.00	11.61
Acetic	13.18	13.16	12.77
Propionic	15.78	15.68	15.43
Butyric	19.79	19.53	19.52
Hippuric	31.31	33.46	31.38

[1] The values for time in seconds are given by integrator in decade system, i.e. 100 equals 60 seconds.

butyric acids were not affected (Table 4.2).

Other normalities of the mobile phase provided the following results: N < 0.02 caused separation of lactic acid from uric and formic acids, but citric and orotic acids coeluted; N > 0.02 provided improved resolution of citric and orotic acids, but caused coelution of lactic, uric and formic acids. Therefore, in some dairy samples, such as cheeses and curds, the organic acid separation was conducted at two normalities of the mobile phase, 0.02 N and 0.0045 N, in order to assess accurately lactic acid in the presence of uric and formic acids and to separate citric from orotic acid.

Organic acids were identified by their retention times in comparison with the retention times of standards and by the spiking method. Quantification was done by peak area integration rather than peak height measurement since the former was more reliable with assymetric peaks of succinic, propionic, butyric and hippuric acids, and avoided errors due to a slight change in pump pressure after extended operation of the instrument.

Uric and formic acids coeluted regardless of the normality of the mobile phase. Hence, their quantification at 220 nm provided only their sum. Therefore, they were rechromatographed at 275 nm, a wavelength at which only the purine ring of uric acid absorbed. Following the recommendation of Marsili *et al.* (1981), the concentrations of both acids were calculated as follows from the peak areas:

$$\text{Area of uric acid in unknown at 220nm} = \frac{\text{Area of uric acid std at 220 nm}}{\text{Area of uric acid std at 275 nm}} \times \text{Area of uric unknown at 275 nm}$$

$$\text{Area of formic acid in unknown at 220nm} = \text{Total area of unknown at 220 nm} - \text{Area of uric unknown at 220 nm}$$

where: std = aqueous standard mixture of uric and formic acids

Detection limits (in μg/ml aqueous solutions) for organic acids analyzed are given in Table 4.3. The highest detection limit of 0.1 μg/ml was found for uric acid, followed by orotic and then hippuric acid. It is obvious that heterocyclic rings of purine and pyrimidine and the benzene ring, respectively, in these acids are responsible for the high detection sensitivity. Pyruvic, citric and lactic acids had medium sensitivity limits, while the dicarboxylic acid (succinic) and the monocarboxylic acid homologues C_1-C_4 had low detection limits ranging from 15-20 μg/ml.

There was a linear correlation between acid concentration and the corresponding peak area. Slopes and intercepts are given in Table 4.4 for the equation of the straight line:

Peak area = y-intercept + slope x acid concentration

Correlation coefficients for peak area vs concentration were

Table 4.3. Detection limits (220nm) for aqueous solutions of organic acid standards by the liquid chromatographic assay as applied in this study.

Acid	Sensitivity (µg/ml)
Citric	4.6
Orotic	0.2
Pyruvic	2.3
Succinic	14.1
Lactic	5.8
Uric	0.1
Formic	15.5
Acetic	16.4
Propionic	18.5
Butyric	20.8
Hippuric	0.2

Table 4.4. Calibration curve data for standard organic acid aqueous dilutions.

Acid	Slope (µg/ml/pa)	y-intercept (µg/ml)	Correlation coefficient
Citric	27.93	-50.25	0.9987
Orotic[1]	982.11	-43.36	0.9999
Pyruvic[2]	104.46	-15.00	0.9999
Succinic	10.78	-5.36	0.9997
Lactic	29.70	-278.11	0.9998
Uric	2854.48	-41.43	0.9999
Formic	21.11	39.89	0.9999
Acetic	11.17	-25.37	0.9999
Propionic	11.19	7.09	0.9999
Butyric	13.17	156.16	0.9997
Hippuric[3]	2384.74	-215.44	0.9998
Uric (275 nm)	2094.05	-61.42	0.9999
Lactic (0.0045 N H_2SO_4)	7.43	-327.72	0.9994

1 Orotic monohydrate was used for the standard curve; results must be multiplied by the factor 0.8966.
2 Sodium pyruvate; multiplication factor 0.8803.
3 Hippuric sodium salt hydrate; multiplication factor 0.8566.

Data apply for the following chromatographic conditions: 20 µl injections; 0.02 N H_2SO_4 as mobile phase; UV detector at 220 nm, sensitivity of Bio-Rad detection system was 0.04; column temperature 65°C; and flow rate 0.7 ml/min [the results for uric (275 nm) and lactic (0.0045 N H_2SO_4) acids have one of the chromatographic conditions different, as shown in brackets].

close to 1, being 0.9999 for most acids, but 0.9998 for lactic and hippuric acids, 0.9997 for succinic and butyric acids, and 0.9987 for citric acid.

Recovery studies were performed with 0.009 N sulfuric acid as mobile phase. All organic acids added to whole milk were recovered over 90%, except for butyric acid (85.6%). These recoveries coincided with those of Marsili *et al.* (1981) for the same LC-system and whole milk samples.

The effect of temperature on separation of organic acids was reflected only by the separation extent of citric and orotic acids. At temperatures of 35°C or below, citric and orotic acids coeluted. They began to resolve at 45°C and higher temperatures, with optimum resolution at 60-65°C; hence 65°C was used in this study. At this temperature an improved retention time for hippuric acid was also obtained, and pyruvic acid, which had closely shouldered the orotic acid peak, was clearly separated.

LC-separation of a mixture of organic acids used as standards is presented in Figure 7.1 (see Appendix). This separation profile was essentially retained with the dairy products analysed (see Appendix, Figures 7.2-7.35, for their chromatograms). All the chromatograms showed a peak with an Rt of 5-6 min due to the solvent acetonitrile, phosphates present in dairy formulations and some unknowns eluting with the void volume. The negative peak recorded at Rt 16-18 min also appeared to be due to acetonitrile since the peak was observed in runs with just acetonitrile and water (Figures 7.2-7.35). Butyric and hippuric acids were eluted as

assymetrical or flattened peaks which often appeared similar to an unstable, noisy base line, as illustrated by the butyric acid peaks in Figures 7.9-7.12.

Moisture contents of dairy products analyzed are given in Table 4.5. The highest moisture content was found in all fluid and cultured products, such as buttermilk or plain yogurt. This was followed by unripened cheeses such as cottage cheese, Quark and Ricotta. The lowest moisture contents were found in ripened cheeses with eyeholes and semi-hard and soft cheeses such as Mozzarella (range 39-52%), mould cheeses (41.5-56%), and textured cheddar cheese (36.5%). Powdered milk (skim milk and buttermilk) had only 4% moisture. As will be described later, the moisture content of the dairy product had a profound influence on the organic acid profile of the product. Moreover, in some dairy products the moisture content correlated at 5% and even at the 1% level with the organic acids present.

The lactose and amino acids in dairy products did not interfere in organic acid detection and quantification. Interference of proteins and most of the polypeptides was avoided by the extraction step, in which acetonitrile precipitated these constituents. Nevertheless, some polypeptides were present in the organic acid extract, as in the case of curd and cheese analysis.

Some polypeptides were found to coelute with succinic acid. This was especially the case with cheeses such as Brie, Camembert, cottage cheese, Edam, Gouda, Mozzarella and Ricotta and with buttermilk and buttermilk powder.

Table 4.5. Moisture contents of some dairy products.

Product		Moisture content (%)
Fluid products	raw milk	87.4
	skim milk	90.5
	2% milk	89.2
	homo milk	88.0
Cream	whipping	56.8
	cereal	81.4
Cultured products	buttermilk	88.0
	natural yogurt	86.3
	sour cream	86.3
Powdered products	skim milk	4.0
	buttermilk	4.0
Lactic curd cheeses	cottage (Canadian)	72.6
	Quark (Canadian)	72.5
Whey cheese	Ricotta (Canadian)	75.0
White brined cheese	Feta (Danish)	52.0
Soft cheeses	Pressed Mozarrella (Canadian)	45.0
	Stretched Mozarrella (")	52.0
Soft surface mould cheeses	Brie (French)	56.0
	Camembert (French)	48.0
	Camembert (Danish)	48.0
	Camembert (Canadian)	55.2
	Marcillat (French)	50.0
Blue veined cheeses	Danablue (Danish)	47.0
	Roquefort (French)	41.5
Textured cheese	Cheddar (Canadian)	36.5
Cheeses with eyeholes	Appenzell (Swiss)	40.0
	Emmental (Swiss)	39.0
	Gruyère (Swiss)	40.0
	Havarti (Danish)	50.0
	Samsoe (Danish)	48.0
Semi-hard cheeses	Edam (Dutch)	41.0
	Gouda (Dutch)	44.0
	Port Salut (French)	42.0

A polypeptide was also found to coelute with propionic acid, as was the case with most of the imported cheeses and some Canadian-made cheeses, such as Camembert, Cheddar, Mozzarella, Quark and Ricotta. In addition this polypeptide was found in cultured buttermilk, yogurt and sour cream. The descending portion of the pyruvic acid peak was shouldered by a polypeptide often encountered in yogurt and some cheeses such as Quark, Ricotta and Camembert.

The joint uric and formic acid peak was also shouldered on its descending portion with a polypeptide in fluid milk products, powders and creams, and cheeses such as Ricotta, Camembert, Brie and cottage cheese.

Lastly, some of the dairy products had two polypeptides preceding the hippuric acid peak with retention times of 26.2 and 30.4 min. These peaks did not interfere in organic acid quantification.

Polypeptide impurities were readily detected by their absorbance at 275 nm, which exceeded that at 220 nm. Their 275/220 nm absorbance ratios were 1.22, 1.43 and 1.50-1.53. Acid hydrolysis of these peaks yielded amino acids, as revealed by thin-layer chromatographic separation followed by detection with ninhydrin reagent.

The precolumn in the LC-system was used to eliminate positively charged molecules such as basic amino acids and other cations present in traces. Dairy lipids (triglycerides, phospholipids and unsaponifiables) did not interfere since they were insoluble in the water-acetonitrile phase of the acid extraction step.

4.2 Fluid and Dehydrated Nonfermented Products and Creams

Results of the analysis of fluid milk and cream are presented in Table 4.6. Fluid milk contained 71.3 ppm orotic acid in homo, 72.1 in raw, and 73.5 in 2% BF milk. The orotic acid content in skim milk was 72.6 ppm. The levels were much lower in creams: 58.7 ppm in 10% BF cereal cream; and 45.4 ppm in 32.5% BF whipping cream.

The orotic acid content of raw milk was close to the 73.3 ppm reported by Lavanty and Steiger (1983), but lower than that found by Marsili *et al.* (1981) and Larson and Hegarty (1979), i.e. 83.6 and 85.3 ppm, respectively, for whole or homo milk. Chen and Larson (1971) reported 67.6-71.4 µg/ml for market milk, which would correspond to 65.7-69.2 ppm by assuming that the specific gravity of their whole milk was 1.032 and of their partly skimmed milk was 1.037.

In the same fluid milk sample the citric acid content ranged from 931.9-960.4 ppm. The highest level, 976.3 ppm, was found in skim milk. The maximal deviation from the mean was ±17 ppm for raw milk but only ±6 ppm for homo or skim milk. In cream the citric acid content was decreased to 782.6 ppm (cereal cream) and to 583.5 ppm (whipping cream). These results agree with literature data of the previously cited authors.

The content of pyruvic acid in fluid milk and cream was generally low. In homo, partially skimmed and skim milk the average was 21.7 ppm, while raw milk had only 12.9 ppm. The content in creams was also lowered to 17.2 ppm in cereal

Table 4.6. Organic acids (ppm) present in some fluid milk products and cream.

	Milk				Cream	
Acid	Raw	Skim	2%	Homo.	Cereal	Whipping
Citric	931.9 ±17.4	976.3 ± 6.5	963.9 ± 3.9	960.4 ± 6.5	782.6 ± 0.6	585.3 ± 0.4
Orotic	72.1 ± 1.0	72.6 ± 0.1	73.5 ± 0.5	71.3 ± 0.2	58.7 ± 0.0	45.4 ± 0.1
Pyruvic[1]	1.5 ± 0.0	19.5 ± 0.6	22.7 ± 0.2	22.8 ± 0.5	17.2 ± 0.2	14.7 ± 0.2
Lactic	<60	<60	<60	<60	n.d.	n.d.
Uric	14.2 ± 0.1	13.7 ± 0.1	13.3 ± 0.1	12.6 ± 0.1	10.1 ± 0.1	8.4 ± 1.8
Hippuric	19.1 ± 0.9	23.5 ± 1.1	18.2 ± 0.9	22.9 ± 1.1	14.0 ± 1.9	10.9 ± 0.7

n.d. = Non-detectable.
[1] Pyruvic acid in freshly milked samples was 1.5 ± 0.02 ppm; in milk pasteurized within three hours of milking the level was 1.5 ± 0.5; and after storage of fresh milk in a cooled tank followed by delivery by bulk tank truck to the dairy plant the level had increased to 15 ppm within 14 hr.

cream and 14.7 ppm in whipping cream.

The present results also revealed the presence of pyruvic acid in all fluid milks and creams. The average content was 22.8 ppm for milk and 14.7-17.2 for creams. Pyruvic acid in raw milk was 1.5±1.0 ppm, however after pasteurization the peak corresponding to this acid provided a result of 70 ppm. Analysis at 275 nm showed absorbance due to a polypeptide, hence pyruvic acid results in all heat-treated milk samples should be considered as inflated.

Lactic acid was detected in all milk samples, but not in creams. Its content was less than 60 ppm. This corroborates the finding of Marsili *et al.* (1981) that lactic acid is present even in fresh milk.

Uric acid content was 12.6-14.2 ppm in fluid milk and 8.4-10.1 ppm in creams. These results are lower than the 21.8 ppm reported by Marsili *et al.* (1981) for commercial milk samples and were closer to 16.5 ppm for raw milk given by Lavanty and Steiger (1984).

Hippuric acid content in fluid milk was 18.2-23.5 ppm and 10.9-14.0 ppm in creams. These results differed (either higher or lower) from those of Marsili *et al.* (1981) and were lower than those given by Lavanty and Steiger (1984) and Patton (1953).

Formic, acetic, propionic and butyric acids were not detected in fluid milk or cream samples. This agreed with the findings of the authors cited above.

4.3 Organic Acid Contents of Fluid and Dehydrated Milk Products

The organic acid profiles of buttermilk powder and skim milk powder, as well as those of their fluid precursors, are shown in Table 4.7. In these products, the major organic acid present was citric acid, ranging from 519.5-1,027.6 ppm in fluid raw milk and skim milk, and from 514.3-534.0 ppm in the buttermilk and skim milk powders. The next most abundant acid was orotic, which ranged from 65.0 ppm in raw milk to 1027.6 ppm in skim milk, while the powders contained 107.9 ppm (buttermilk) and 108.1 ppm (skim milk). Concentrations of both orotic and uric acids were higher in fluid buttermilk and skim milk, compared to whole raw milk. Citric acid content was higher in liquid buttermilk and skim milk than in their respective powders. It is probable that in spray-drying (215°C) a portion of the citric acid was thermally degraded. At temperatures above 175°C, citric acid is partially converted (by dehydration) to aconitic acid, and partially to itaconic acid (by decarboxylation and dehydration).

Pyruvic acid content was, as expected, lowest in liquid buttermilk (19.2 ppm) and highest in buttermilk powder (80.5 ppm), while this trend was reversed in the case of skim milk: in fluid skim milk it was 78.2 ppm and in powder 63.2 ppm. However, the content in powder was still much lower than that required solely by the concentration effect, which strongly suggested its thermal decomposition. As known, pyruvic acid decomposes at its boiling point (165°C).

Table 4.7. Organic acids (ppm) present in some fluid and dehydrated milk products.

Acid	Raw milk	Buttermilk	Skim milk	Buttermilk powder	Skim milk powder
Citric	519.5 ±17.1	995.6 ± 3.6	1,027.6 ± 6.0	514.3 ±20.8	534.0 ± 5.9
Orotic	65.0 ± 0.6	76.7 ± 0.5	79.6 ± 0.3	107.9 ± 0.2	108.1 ± 2.7
Pyruvic	52.9 ± 0.3	19.2 ± 0.3	78.2 ± 0.3	80.5 ± 0.0	63.2 ± 2.3
Lactic	<60	<60	<60	<60	<60
Uric	25.0 ± 0.1	19.4 ± 0.4	29.3 ± 0.2	22.7 ± 0.1	30.4 ± 0.4
Acetic	n.d.	n.d.	n.d.	332.5 ± 1.8	n.d.
Propionic	n.d.	n.d.	n.d.	347.4 ± 9.0[1]	n.d.
Butyric	<50	---[2]	<50[1]	---	250 ± 40
Hippuric	20.2 ± 0.8	---	25.7 ± 1.2	5.5 ± 0.7	85.9 ± 1.4

[1] This result is probably quite inflated due to propionic acid coelution with a polypeptide.
[2] "---" in this and following tables indicates not determined due to flattened peaks and base line noise.

Relatively large amounts of acetic and propionic acids in buttermilk powder cannot be explained unless microbial contamination occurred in the process prior to the spray-drying step.

The uric acid content did not change significantly in skim milk spray-drying. Hippuric acid was present in all of these products at a level of 20.2-85.2 ppm. In buttermilk it was not detectable, while in buttermilk powder it was only 5.5 ppm.

Compared to the results for skim milk powder reported by Marsili *et al.* (1981), our results showed lower amounts of citric, orotic, uric and hippuric acids, but higher amounts of pyruvic acid. Davidson (1984) reported 200-210 ppm of lactic acid in fresh milk powder; while Marsili *et al.* (1981) found values lower than 150 ppm, much closer to the results of this study (<60 ppm). Larsen and Hegarty (1979) reported high concentrations of orotic acid in evaporated milk (165 ppm) or dried skim milk (802 ppm). The latter result is still less than half of the theoretical value expected by solely concentrating the fluid skim milk (moisture content 90.5%) to powder (moisture content 4%).

4.4 Organic Acid Contents of Fermented Dairy Products

The organic acids in cultured dairy products were followed by processing steps in cultured buttermilk, sour cream and yogurt production.

4.4.1 Cultured buttermilk

Table 4.8 presents data for organic acids in cultured buttermilk, which was prepared using a starter culture consisting of *Lactobacillus cremoris* and *Streptococcus cremoris* and/or *lactis*.

The dominant acid generated during incubation was lactic acid. Immediately after inoculation its level was 190.6 ppm; it then started to rise, and after 17.5 hr reached its highest level, 4,252.5 ppm (see Figure 4.1). When the incubation (23°C) was terminated and the product cooled to 4-5°C, there was still a residual microbial acitivity augmenting this result by an 179 ppm of lactic acid. Next in abundance was acetic acid. While it was not present in milk before inoculation, it was found after 2 hr of incubation (40.3 ppm). This amount increased until the end of incubation by 8.6-fold, and reached its maximum in cooled end-product (540.1 ppm). This value is lower than 850 ppm reported by Marsili *et al.* (1981) for the same commercially prepared product.

Citric acid, the third major acid of cultured buttermilk, was present at 257.6 ppm. This content is 23% of the original content in milk utilized for buttermilk production. The citric acid decrease was accompanied by a decrease in orotic acid content. Both acids are utilized in the metabolism of the starter culture. The slight increase in pyruvic acid content appeared to be related to lactic acid biosynthesis. Lastly, during incubation propionic acid was formed, reaching its maximum of 63.8 ppm after the end

Table 4.8. Organic acids (ppm) present at various stages of cultured buttermilk production.

Acid	Before inoculation	After inoculation	Incubation time (hr)		
			2	4.5	7.5
Citric	1,108.6 ±19.5	1,035.4 ± 6.1	1,027.6 ± 5.8	987.6 ±11.9	743.6 ± 0.0
Orotic	82.0 ± 1.6	81.1 ± 0.2	80.1 ± 0.1	79.2 ± 1.0	79.7 ± 0.4
Pyruvic	21.0 ± 0.1	20.0 ± 0.7	20.2 ± 2.8	18.5 ± 2.8	22.4 ± 2.8
Lactic	<60	190.6 ± 7.6	202.7 ± 5.4	381.5 ± 7.7	826.5 ±39.9
Uric	28.6 ± 0.5	21.8 ± 0.0	24.5 ± 0.2	22.4 ± 0.0	25.9 ± 0.1
Acetic	n.d.	n.d.	40.3 ± 1.2	57.1 ± 2.3	105.1 ± 4.3
Propionic[1]	n.d.	n.d.	n.d.	14.9 ± 7.4	40.9 ± 5.6

[1] Due to propionic acid coelution with an unknown polypeptide, the results were reported as $A_{...}$-$A_{...}$; when this was omitted, propionic acid results were inflated by a factor of 2.

(cont.)

Table 4.8 (cont.). Organic acids (ppm) present at various stages of cultured buttermilk production.

Acid	Incubation time (hr)				Finished product
	10.5	13.5	16.5	17.5	
Citric	403.8 ± 6.7	363.4 ±34.7	288.8 ±12.3	247.5 ±14.9	257.6 ± 3.7
Orotic	62.2 ± 0.1	54.2 ± 1.2	52.2 ± 0.1	52.0 ± 0.7	50.3 ± 0.2
Pyruvic	28.3 ± 0.8	33.9 ± 0.6	46.6 ± 0.8	47.8 ± 0.7	40.2 ± 1.8
Lactic	2,315.1 ± 0.3	3,811.4 ± 7.6	4,156.1 ±33.2	4,252.5 ±71.6	4,431.7 ±47.2
Uric	30.2 ± 0.1	30.3 ± 0.1	29.6 ± 0.4	30.8 ± 0.2	27.9 ± 0.3
Acetic	136.1 ± 1.2	190.6 ± 7.1	307.3 ± 6.4	346.8 ± 7.5	540.1 ±19.7
Propionic	50.8 ± 0.1	60.4 ± 6.6	61.6 ± 4.6	63.8 ± 3.3	61.5 ± 6.4

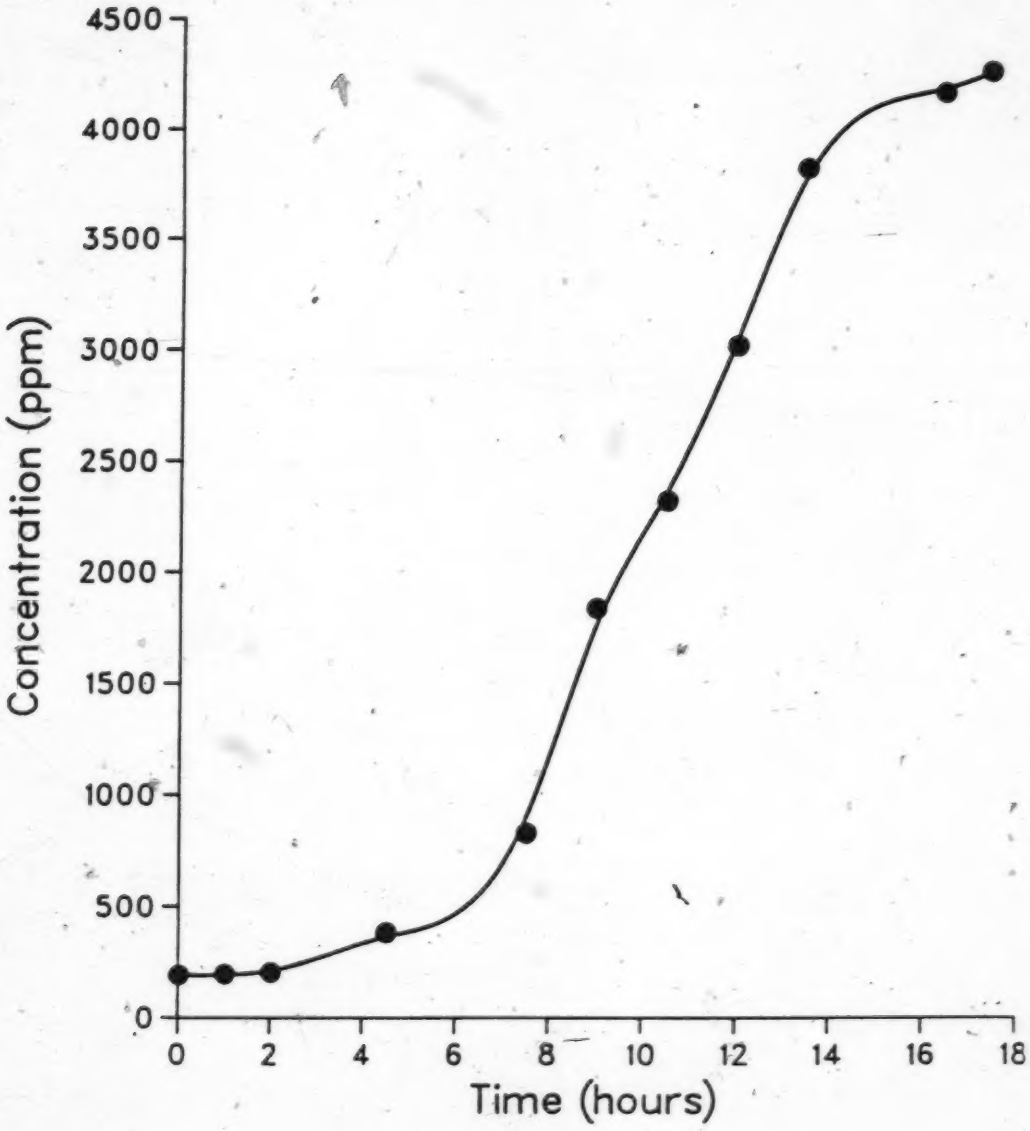

Figure 4.1 Lactic acid formation (ppm) during incubation of milk in the production of cultured buttermilk.

of the incubation period. For a similar product, the only data available in the literature was that of Marsili *et al.* (1981): less than 60 ppm.

4.4.2 Sour cream

Sour cream data are provided in Table 4.9. It was prepared from a blend of skim milk and cream with the addition of skim milk powder and gelatin as thickener. Here, the fermentation time, using the same starter culture as buttermilk, was 12.5 hr, i.e., 4 hr shorter than with cultured buttermilk.

Again, lactic acid was the dominant acid. At the end of incubation it had reached 2,957 ppm, a level which was practically retained by cooled end-product. The rise of lactic acid content during incubation is given in Figure 4.2. Next to lactic acid in content was that of citric acid. As in other cultured dairy products, its level decreased with incubation time from 863.9 before inoculation to 349.8 ppm in the end-product. This decrease during incubation was also found with orotic acid. Similar again to buttermilk, the slight increase in pyruvic acid was related to an enhanced biosynthesis of acetic and propionic acids, both reaching a level less than 70 ppm.

Literature data for sour cream orotic acid content were 48.6 (Marsili *et al.*, 1981) and 45.4 ppm (Lavanty and Steiger, 1984); while for acetic and propionic acids the values were 900 and 180 ppm (Marsili *et al.*, 1981) and 880 and 175 ppm, respectively (Lavanty and Steiger, 1984). It

Table 4.9. Organic acids (ppm) present at various stages of sour cream production.

Acid	Before inoculation	After inoculation	Incubation time (hr)				Finished product
			2	6	9	12.5	
Citric	863.9 ± 5.2	808.4 ±26.6	623.2 ±23.8	423.9 ± 9.3	351.3 ± 8.8	370.3 ± 3.2	349.8 ±25.1
Orotic	73.2 ± 0.1	71.2 ± 1.4	65.5 ± 1.5	51.2 ± 0.2	48.8 ± 0.5	49.6 ± 0.2	46.0 ± 0.9
Pyruvic	18.6 ± 3.1	27.4 ± 2.5	29.4 ± 0.3	27.9 ± 0.7	27.3 ± 2.4	34.8 ± 1.3	35.8 ± 2.5
Lactic	<60	232.1 ±10.0	819.5 ±12.2	2,504.8 ± 1.0	2,897.1 ± 2.1	2,957.8 ±18.2	3,031.8 ± 3.2
Uric	11.5 ± 0.4	12.5 ± 0.2	12.0 ± 0.2	14.1 ± 0.1	13.6 ± 0.1	12.6 ± 0.4	11.9 ± 0.4
Acetic	n.d.	n.d.	64.3 ± 0.4	64.5 ± 1.5	66.7 ± 0.2	67.0 ± 1.2	66.6 ± 0.3
Propionic	n.d.	n.d.	52.9 ± 0.1	53.8 ± 1.3	57.2 ± 1.8	61.7 ± 0.5	60.9 ± 1.9

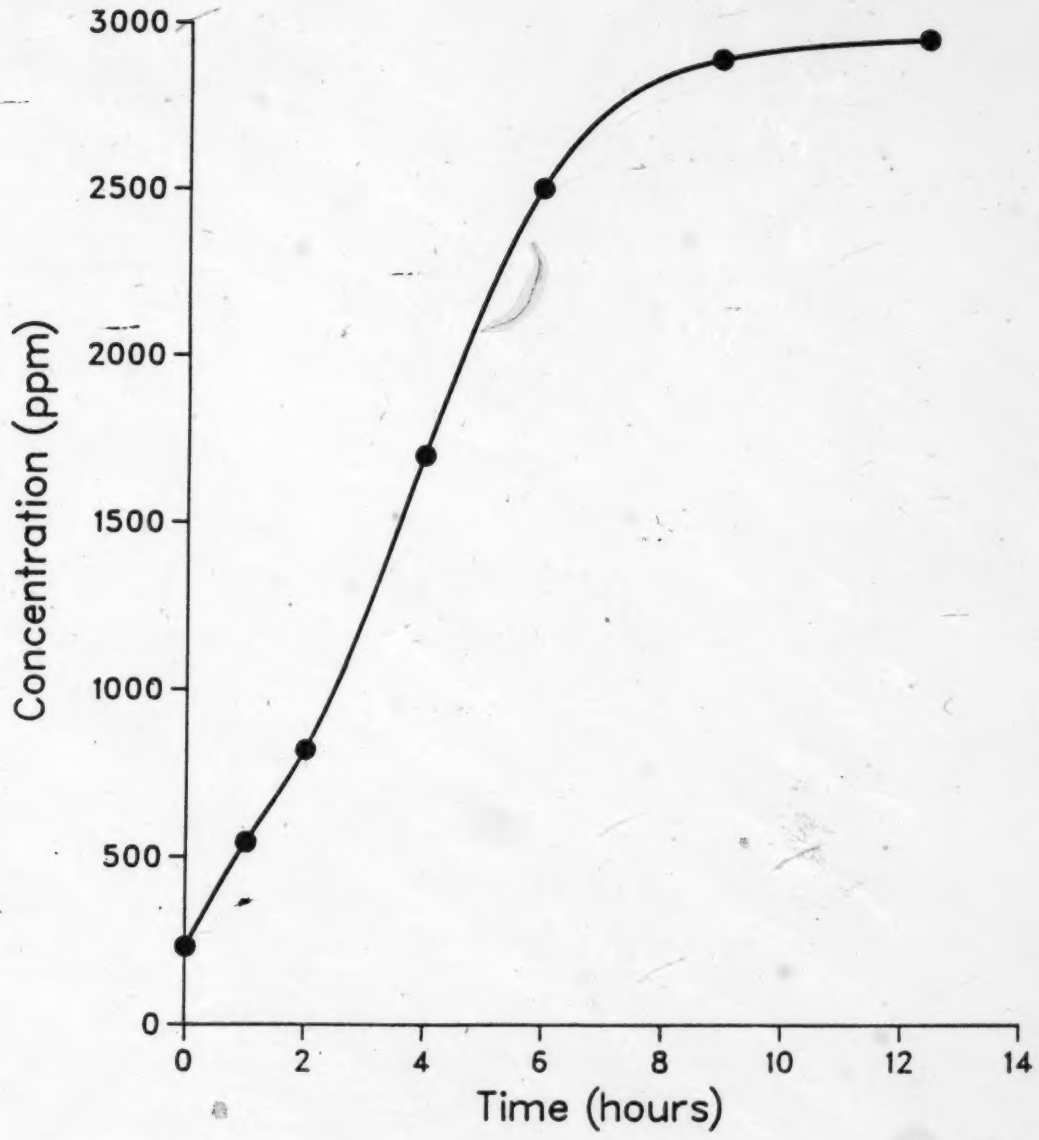

Figure 4.2 Lactic acid formation (ppm) during incubation of milk in the production of sour cream.

appears that these authors had a sour cream process different from the one given in this study. This suggestion is supported by their data for lactic acid content, which ranged from 8,410-9,215 ppm.

4.4.3 Yogurt

The formation of organic acids in a yogurt process is shown in Table 4.10. In this process, milk was inoculated with *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. It was incubated for 6 hr at 43°C, and then cooled to 4-5°C. The yogurt end-product contained 7,955.2 ppm lactic acid, the major organic acid in the product. During yogurt production lactic acid increased by 132.6 times the original acid content in fluid milk (see Figure 4.3). Thus, its content is much higher than that in sour cream and cultured buttermilk. The rate of biosynthesis of most organic acids was more rapid than in other cultured dairy products, although the incubation time for yogurt was shorter.

The second most abundant acid was citric acid. Its content decreased during incubation from 990.8 to 693.0 ppm (the level found in the end-product). Simultaneously, orotic acid decreased from 99.3 to 25.9 ppm. On the other hand, there was an increase in the content of pyruvic acid from 22.9 to 43.2 ppm. Propionic and acetic acids in the end-product were 132.7 and 95.8 ppm, respectively. Hippuric acid content was 4.3 ppm, which is much lower than found in fluid milk samples. Lastly, in the yogurt process there was

Table 4.10. Organic acids (ppm) present at various stages of yogurt production.

Acid	Before inoculation	After inoculation	Incubation time (hr) 1	Incubation time (hr) 2
Citric	990.8 ±20.2	990.5 ±18.7	979.6 ± 2.1	630.2 ± 2.4
Orotic	99.3 ± 1.1	101.0 ± 0.7	98.4 ± 0.5	86.6 ± 1.6
Pyruvic	22.9 ± 0.2	25.5 ± 2.2	28.8 ± 0.0	30.3 ± 0.8
Lactic	<60	267.7 ± 0.1	772.7 ± 1.6	2,545.4 ±53.8
Uric	24.3 ± 0.3	25.6 ± 0.2	25.8 ± 0.0	29.0 ± 0.4
Acetic	n.d.	57.6 ± 1.5	68.0 ± 3.2	66.8 ± 4.1
Propionic[1]	n.d.	8.4 ± 0.8	25.8 ± 1.8	45.6 ± 2.2

[1] Due to propionic acid coelution with an unknown polypeptide, the results were calculated from A_{215}-A_{225}; if this was omitted, the propionic acid results in this and following tables were inflated by a factor of 10. *(cont.)*

Table 4.10 (cont.). Organic acids (ppm) present at various stages of yogurt production.

Acid	Incubation time (hr) 3	Incubation time (hr) 4	Incubation time (hr) 6	Finished product
Citric	585.2 ± 0.3	598.4 ±13.7	696.3 ±11.0	693.0 ±11.1
Orotic	64.0 ± 0.1	46.8 ± 0.6	36.8 ± 0.2	25.9 ± 0.4
Pyruvic	35.6 ± 0.0	37.7 ± 0.2	40.1 ± 0.4	43.2 ± 0.3
Lactic	5,055.8 ±17.3	6,369.2 ±71.2	7,208.4 ±43.4	7,955.2 ±30.4
Uric	31.7 ± 0.1	30.7 ± 1.0	32.5 ± 0.1	35.3 ± 0.1
Acetic	66.8 ± 4.1	87.3 ± 6.6	95.7 ± 1.7	95.8 ± 0.4
Propionic	45.6 ± 2.7	51.2 ± 1.7	107.8 ± 1.9	132.7 ± 5.1
Hippuric	---	---	---	4.3 ± 0.3

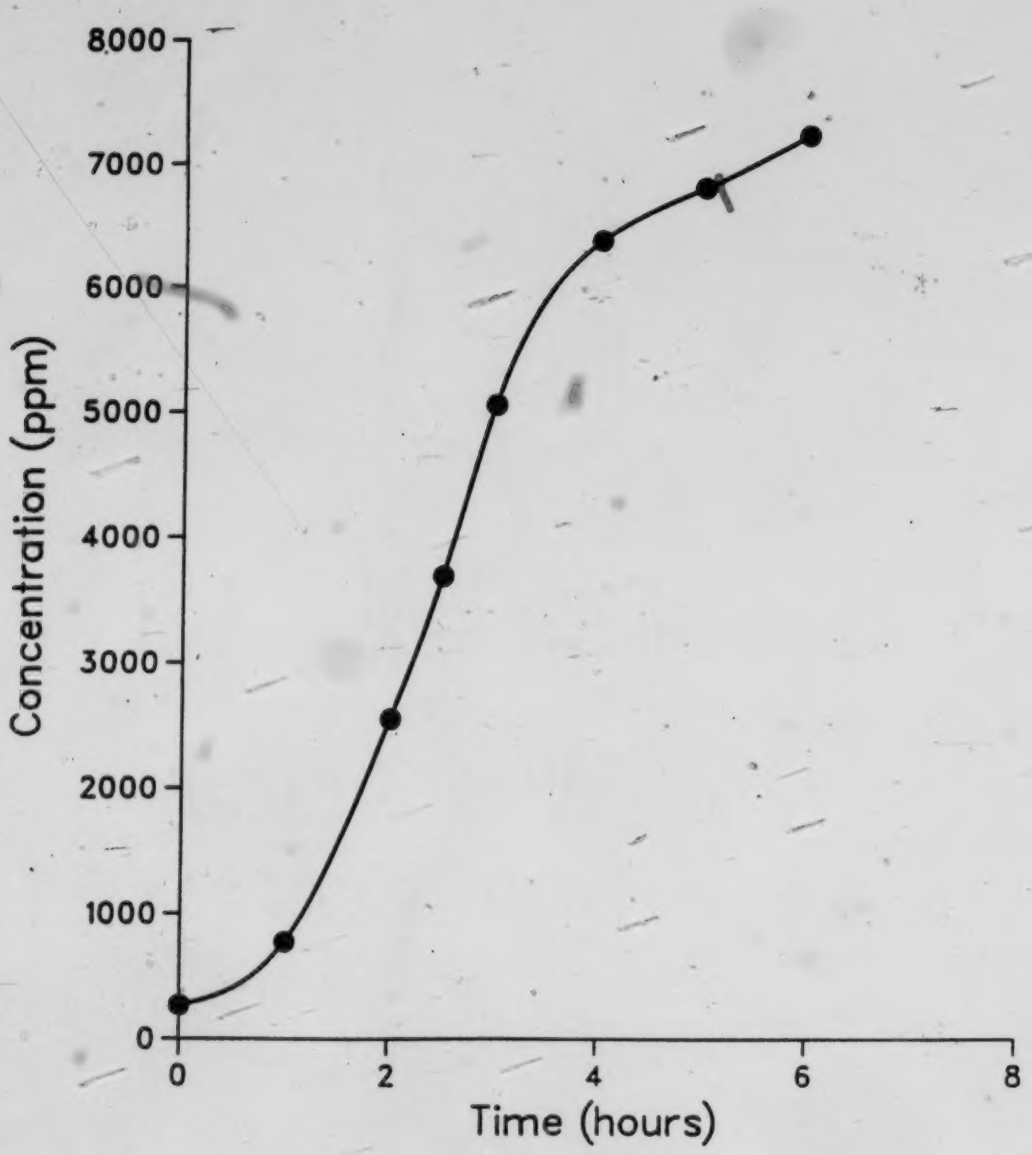

Figure 4.3 Lactic acid formation (ppm) during incubation of milk in the production of yogurt.

a slight (1.45%) increase in the content of uric acid.

Haggerty *et al.* (1984) reported a 15-53% decrease in orotic acid content after fermentation in the yogurt process, but did not find an increase in uric acid content. In the end-product they reported 17.3-32.4 ppm of orotic and 14.2-18.2 ppm of uric acid. Rasic and Kurman (1978) detected in the yogurt process a relative decrease in citric acid of 25-30%, though its absolute level remained unchanged. They also reported a considerable decrease in orotic acid due to its consumption by lactic acid bacteria, particularly *Lactobacillus bulgaricus*. Lastly, both Marsili *et al.* (1981) and Lavanty and Steiger (1984) found slightly higher values for citric acid (710-725 ppm) than found in this study, as well as higher contents of orotic acid (72.5-75.5 ppm). However, their data for lactic acid content in yogurt (14,550 and 16,280 ppm, respectively) are nearly double that found in this study. The result of Ashoor and Wetty (1984), 12,080 ppm, was closer to the data of this study. The literature data for acetic acid content in yogurt are close to that given in this study.

4.5 Organic Acid Contents of Cheeses

4.5.1 Domestic cheeses

4.5.1.1 Camembert

Camembert cheese was produced by the addition of both lactic acid bacteria and *Penicillium* white mould to the milk. Table 4.11 shows that lactic acid was the dominant

Table 4.11. Organic acids (ppm) present at various stages of Camembert cheese production.

Acid	Raw milk	Pasteurized milk	Addition of		Cutting	After cutting	
			culture	rennet		whey	curd
Citric	1,124.0 ±34.2	1,153.6 ±28.4	1,131.4 ±31.9	1,160.9 ± 3.5	1,069.6 ± 8.7	1,484.7 ±35.3	383.9 ±22.3
Orotic	79.7 ± 2.2	80.4 ± 2.1	78.5 ± 2.2	78.3 ± 0.3	76.3 ± 0.0	81.5 ± 0.1	4.4 ± 0.1
Pyruvic	33.0 ± 1.7	33.8 ± 1.2	32.4 ± 0.5	34.4 ± 0.6	36.7 ± 1.9	39.2 ± 0.5	15.4 ± 0.4
Lactic	<60	<60	324.3 ± 0.1	436.1 ±36.3	455.5 ± 0.5	499.3 ±10.3	394.3 ± 4.2
Uric	17.7 ± 0.0	16.8 ± 0.1	16.4 ± 0.2	14.8 ± 0.2	16.8 ± 0.0	14.1 ± 0.4	12.2 ± 0.0
Acetic	n.d.	n.d.	n.d.	64.4 ± 0.8	64.6 ± 0.4	71.1 ± 0.8	53.3 ± 0.1
Propionic	n.d.	n.d.	n.d.	6.2 ± 0.2	8.5 ± 0.7	12.7 ± 0.4	13.1 ± 1.3

(cont.)

Table 4.11 (cont.). Organic acids (ppm) present at various stages of Camembert cheese production.

Acid	After moulding (pH = 4.4, 9 hr later)	Finished product, next day	Ripening stage (days)				
			5	18	26	33	40
Citric	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Orotic	3.2 ± 0.1	3.2 ± 0.1	2.5 ± 0.2	1.9 ± 0.2	1.8 ± 0.2	2.2 ± 0.3	1.8 ± 0.2
Pyruvic	19.0 ± 0.2	18.7 ± 0.3	14.6 ± 0.3	14.9 ± 0.6	10.9 ± 0.1	11.9 ± 0.4	9.8 ± 0.0
Lactic	607.3 ±30.7	1,184.8 ± 6.2	1,706.0 ±87.9	1,849.9 ±13.1	1,771.5 ±12.4	1,667.8 ±23.0	1,730.3 ± 0.7
Uric	5.8 ± 0.0	8.4 ± 0.0	9.2 ± 0.1	5.7 ± 0.0	5.6 ± 0.1	9.3 ± 0.1	5.3 ± 0.2
Acetic	53.4 ± 0.8	52.9 ± 0.3	54.0 ± 0.3	53.3 ± 1.1	51.4 ± 1.3	53.0 ± 1.3	49.6 ± 0.5
Propionic	18.4 ± 0.4	21.6 ± 0.9	21.4 ± 0.8	20.2 ± 0.2	18.6 ± 1.1	22.7 ± 1.7	29.3 ± 0.1

acid and that its content increased from under 60 ppm in the raw milk to 1,730.3 ppm in Camembert after 40 days of ripening. Simultaneously, acetic acid increased sharply after culture addition, and after a slight decrease, it levelled off at 49.6 ppm in the end-product. Propionic acid increased steadily after culture addition, and in the end-product was 29.3 ppm. Citric, orotic, pyruvic and uric acids were the minor acids present, decreasing markedly during the process, and for the most part were removed into the whey fraction. After moulding, whey drainage still occurred and as a result citric acid could not be detected. Orotic acid concentration was low and was 1.8 ppm in the end product, i.e., 97.7% less than its concentration in raw milk. Pyruvic acid decreased by 70.3% during the process, with 9.8 ppm in the end-product. Uric acid decreased to 5.3 ppm in cheese after ripening, i.e., a decrease of 70%.

4.5.1.2 Cottage cheese

Organic acid levels in cottage cheese production are given in Table 4.12. The addition of starter culture *Lactobacillus cremoris* and *Streptococcus cremoris* and/or *lactis* caused a profound change in acid levels. The major change was in the content of lactic acid. As seen in Figure 4.4, after approximately 30 min of lag phase (during milk incubation) there is an exponential rise in lactic acid formation. While in the fresh milk the lactic acid content was <60 ppm, in the lag phase it was 500 ppm, and at the end of the incubation period (5.5 hr) the acid formation levelled off at a content of 3,849.8 ppm.

Table 4.12. Organic acids (ppm) present at various stages of cottage cheese production.

Acid	Skim milk	Cheese milk[1]	Culture addition	Rennet addition	Incubation time (hr)	
					1	2
Citric	1,019.7 ± 8.4	991.4 ±17.5	1,023.7 ± 3.4	1,009.7 ± 2.8	984.5 ± 6.6	996.2 ± 7.9
Orotic	72.1 ± 0.2	79.5 ± 1.3	80.9 ± 0.1	80.2 ± 0.1	81.3 ± 0.4	78.3 ± 0.2
Pyruvic	28.4 ± 0.5	30.8 ± 0.5	31.2 ± 0.8	26.6 ± 0.2	26.7 ± 0.2	27.1 ± 0.1
Lactic	<60	<60	459.0 ± 3.1	469.3 ± 4.8	629.7 ± 6.6	1,042.2 ± 6.4
Uric	13.4 ± 0.1	14.3 ± 0.3	15.0 ± 0.3	14.4 ± 0.4	14.3 ± 0.2	14.4 ± 0.1
Hippuric	14.3 ± 0.7	---	---	---	---	---

[1] Cheese milk is skim milk with skim milk powder and mineral supplement added.

(cont.)

Table 4.12 (cont.). Organic acids (ppm) present at various stages of cottage cheese production.

Acid	Incubation time (hr)				Cutting stage	
	3	4	5	5.5	curd	whey
Citric	977.5 ± 4.4	971.2 ± 1.0	979.6 ±12.9	977.1 ± 6.9	363.3 ± 2.6	1,103.1 ±15.2
Orotic	73.7 ± 0.0	64.0 ± 0.2	60.8 ± 0.5	60.9 ± 0.2	49.5 ± 0.0	61.8 ± 0.7
Pyruvic	26.9 ± 0.1	26.9 ± 0.1	22.2 ± 0.6	22.4 ± 0.4	19.9 ± 0.0	22.4 ± 0.2
Lactic	1,764.4 ± 3.5	2,927.3 ± 4.3	3,762.5 ±45.8	3,849.8 ±53.0	3,138.6 ± 4.1	3,983.6 ±14.7
Uric	14.0 ± 0.2	13.8 ± 0.2	13.2 ± 0.3	13.2 ± 0.1	11.0 ± 0.1	12.8 ± 0.1

(cont.)

Table 4.12 (cont.). Organic acids (ppm) present at various stages of cottage cheese production.

Acid	Curd at cooking	Whey at drainage	Dry curd at drainage	Cream	Finished product
Citric	352.5 ±20.4	1,130.9 ± 4.1	84.4 ± 1.2	804.0 ± 0.8	279.0 ± 5.4
Orotic	50.4 ± 1.1	62.7 ± 0.5	13.0 ± 0.1	61.0 ± 0.6	29.1 ± 0.0
Pyruvic	24.6 ± 2.1	47.1 ± 0.1	6.4 ± 0.4	26.9 ± 0.3	11.1 ± 0.4
Lactic	3,462.9 ±18.6	4,119.5 ± 9.0	671.4 ± 3.6	n.d.	449.4 ± 6.1
Uric	7.1 ± 0.1	12.8 ± 0.2	3.9 ± 0.3	10.2 ± 0.2	7.2 ± 0.1

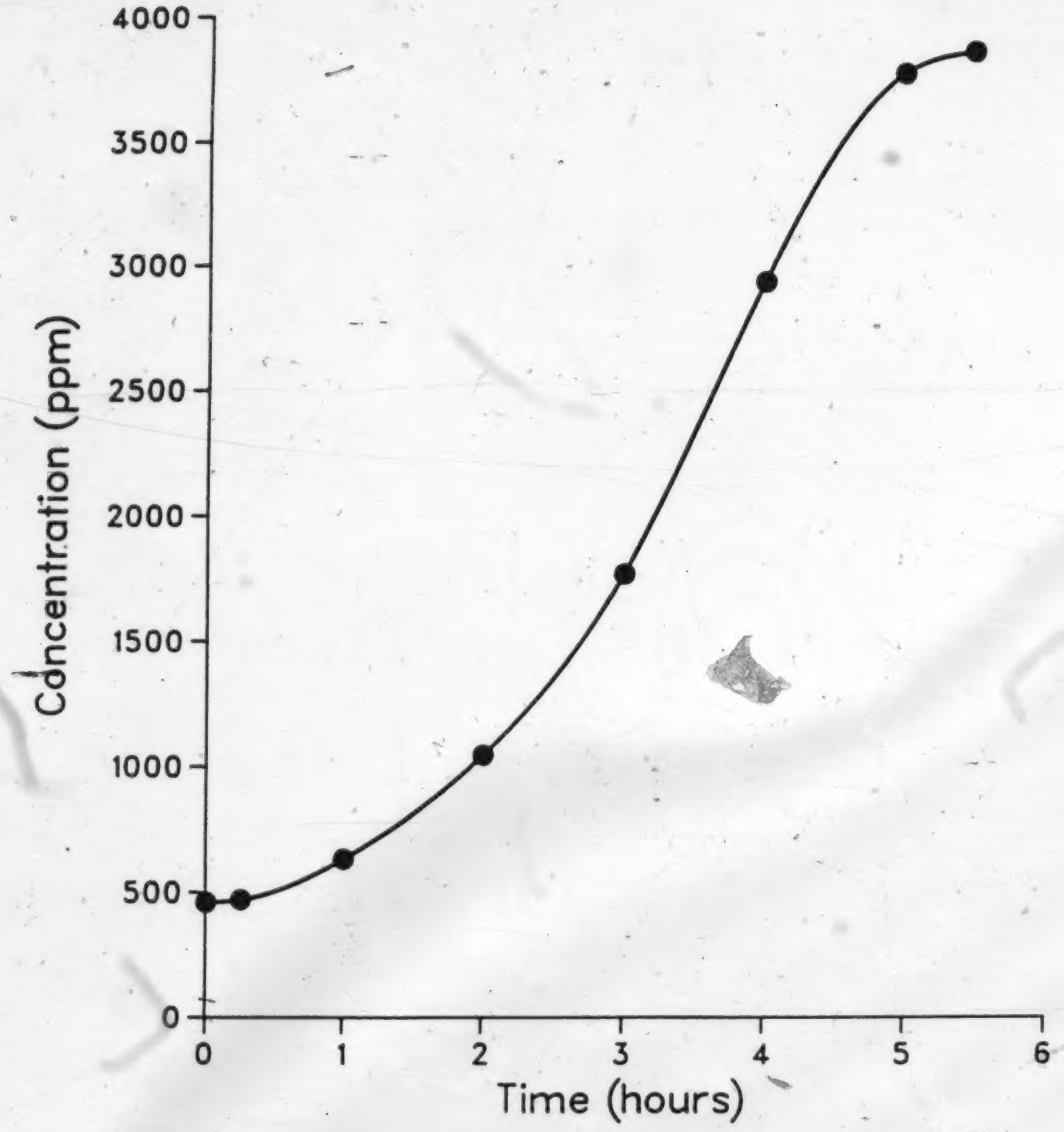

Figure 4.4 Lactic acid formation (ppm) during incubation of milk in the production of cottage cheese.

During incubation there was only a small drop in the level of pyruvic acid. It is obvious that pyruvic acid did not accumulate during lactose fermentation by starter cultures, but rather retained the role of a steady-state intermediary product in its reduction to lactic acid. The slight decrease in citric, orotic and uric acids during the incubation period might be accounted for by their utilization in the metabolism of the starter culture.

Formic, acetic, propionic and butyric acids were not present in any stage of cottage cheese production. Since they might serve as markers for spoilage organisms, their absence in cottage cheese indicates the utilization of pure, non-contaminated starter cultures.

The dominant acid in cottage cheese was lactic acid. Its level was only 671.4 ppm at the curd stage, with the major portion being removed by whey drainage. After adding the cream to curd to a 2% BF content, the lactic acid level of the end-product was 449.4 ppm. Next in abundance in cottage cheese end-product was citric acid. Its level was 279 ppm, lower than levels reported for commercial cottage cheese by Marsili *et al.* (1981). The same was the case for orotic acid: its value in our product was 29.1 ppm, while the values given by Marsili *et al.* (1981) and Larson and Hegarty (1979) were 39.0 and 39.8, respectively.

4.5.1.3 Cheddar cheese

The organic acid contents of two batches, designated A and B, of Cheddar cheeses are shown in Tables 4.13 and 4.14. The batches were made on the same day in the same plant and

Table 4.13. Organic acids (ppm) present at various stages of Cheddar cheese production (batch A).

Acid	Raw milk	Pasteurized milk		Culture addition	Rennet addition	Cutting	Heating (start)
		line	tank				
Citric	878.4 ±19.6	863.4 ± 1.6	857.1 ± 1.9	865.2 ±12.2	883.0 ± 3.5	861.4 ±12.6	876.7 ± 5.0
Orotic	72.2 ± 0.0	73.7 ± 0.1	71.6 ± 0.0	71.1 ± 0.2	70.5 ± 0.4	71.2 ± 0.9	72.8 ± 0.1
Pyruvic	23.4 ± 0.4	23.0 ± 0.3	21.5 ± 0.2	15.0 ± 2.2	17.5 ± 0.4	15.6 ± 0.2	19.8 ± 1.6
Lactic	<60	<60	<60	262.5 ±24.3	243.0 ± 1.2	278.3 ± 2.0	294.0 ± 4.1
Uric	25.2 ± 0.7	25.9 ± 0.1	25.1 ± 0.2	23.2 ± 0.4	22.2 ± 0.3	21.9 ± 0.0	23.1 ± 0.2
Acetic	n.d.	n.d.	n.d.	73.6 ± 3.3	72.0 ± 4.1	75.5 ± 1.9	74.9 ± 3.4
Propionic	n.d.	n.d.	n.d.	6.5 ± 0.2	6.1 ± 0.1	6.2 ± 0.2	9.5 ± 0.1

(cont.)

Table 4.13 (cont.). Organic acids (ppm) present at various stages of Cheddar cheese production (batch A).

Acid	Heating (end)	Before drainage	Dry curd (piled)	Whey at piling	Milling	Moulding and salting	Pressing
Citric	862.8 ± 9.4	870.0 ±12.1	23.7 ± 2.9	1153.9 ±18.8	23.3 ± 0.6	27.4 ± 1.7	18.5 ± 2.4
Orotic	73.9 ± 0.0	60.4 ± 0.7	19.7 ± 0.0	64.0 ± 0.0	14.9 ± 0.3	15.1 ± 0.5	12.7 ± 0.2
Pyruvic	17.4 ± 0.7	14.2 ± 2.0	7.3 ± 1.8	22.0 ± 1.2	13.1 ± 0.3	12.7 ± 0.6	11.6 ± 0.4
Lactic	384.7 ±17.1	644.8 ± 4.8	1,398.2 ±10.4	1,727.6 ± 0.5	2,433.2 ±18.4	2,640.6 ±68.2	3,071.5 ±64.8
Uric	22.9 ± 0.4	19.1 ± 0.0	7.4 ± 0.1	19.6 ± 0.1	5.3 ± 0.2	4.0 ± 0.1	3.2 ± 0.1
Acetic	165.2 ± 2.6	295.4 ± 2.9	239.3 ± 0.2	405.7 ± 6.7	263.6 ± 5.1	250.1 ± 0.8	242.1 ± 5.2
Propionic	6.8 ± 0.3	18.2 ± 0.3	18.2 ± 0.7	12.6 ± 0.4	18.0 ± 0.8	18.1 ± 0.3	18.5 ± 1.2

(cont.)

Table 4.13 (cont.). Organic acids (ppm) present at various stages of Cheddar cheese production (batch A).

	Ripening stage (days)						
Acid	19	43	69	92	118	133	174
Citric	20.4 ± 3.5	22.8 ± 1.9	24.2 ± 1.6	22.5 ± 2.9	19.4 ± 1.2	18.9 ± 1.0	20.8 ± 2.3
Orotic	8.8 ± 0.4	8.1 ± 0.0	8.9 ± 0.2	9.3 ± 0.0	7.5 ± 0.4	9.4 ± 0.1	8.8 ± 0.1
Pyruvic	11.1 ± 0.9	8.6 ± 0.8	10.6 ± 1.0	10.4 ± 1.1	7.8 ± 1.2	11.2 ± 2.1	9.8 ± 0.2
Lactic	4,197.9 ±160.2	4,095.1 ±13.8	4,224.0 ±23.9	4,310.1 ±15.2	4,150.4 ±10.3	4,409.5 ±10.2	4,430.6 ± 5.5
Uric	1.8 ± 0.0	4.2 ± 0.2	2.3 ± 0.4	1.1 ± 0.2	0.3 ± 0.1	0.5 ± 0.0	0.4 ± 0.0
Acetic	255.7 ± 3.1	277.4 ± 9.0	266.1 ± 3.1	291.3 ± 5.3	254.6 ± 3.0	272.0 ± 2.3	290.4 ±17.9
Propionic	17.4 ± 0.9	15.8 ± 0.8	16.4 ± 0.1	17.3 ± 0.2	17.4 ± 0.5	17.2 ± 0.2	18.8 ± 1.1

Table 4.14. Organic acids (ppm) present at various stages of Cheddar cheese production (batch B).

Acid	Raw milk	Culture addition	Rennet addition	Cutting	Heating	
					start	end
Citric	894.6 ± 1.0	878.8 ±35.5	883.4 ± 0.9	882.3 ± 1.7	873.7 ± 8.0	883.6 ± 7.4
Orotic	62.6 ± 0.5	69.8 ± 0.5	70.4 ± 0.0	70.4 ± 0.2	71.4 ± 0.2	74.1 ± 0.6
Pyruvic	19.2 ± 1.4	19.4 ± 1.1	19.1 ± 0.2	17.9 ± 1.4	18.6 ± 1.3	18.5 ± 3.2
Lactic	<60	235.8 ± 3.7	244.7 ± 1.5	313.5 ± 1.1	338.1 ±11.1	431.3 ± 3.8
Uric	22.6 ± 0.2	24.4 ± 0.0	24.2 ± 0.2	22.3 ± 0.2	22.6 ± 0.1	23.3 ± 0.0
Acetic	n.d.	27.5 ± 0.1	70.9 ± 4.9	83.0 ± 1.3	101.9 ± 1.5	138.1 ±13.0
Propionic	2.2 ± 0.1	2.8 ± 0.1	7.0 ± 0.2	7.8 ± 0.1	9.3 ± 0.1	9.3 ± 0.1

(cont.)

Table 4.14 (cont.). Organic acids (ppm) present at various stages of Cheddar cheese production (batch B).

Acid	Before drainage	Dry curd (piled)	Whey at piling	Milling	Moulding and salting	Pressing
Citric	865.2 ± 4.0	25.4 ± 0.5	989.6 ±19.1	24.0 ± 1.1	20.8 ± 0.9	20.1 ± 0.7
Orotic	60.9 ± 0.3	19.5 ± 0.2	65.7 ± 0.2	13.9 ± 0.2	15.0 ± 0.0	11.8 ± 0.1
Pyruvic	16.2 ± 0.4	5.5 ± 0.2	19.2 ± 0.5	8.7 ± 0.3	10.2 ± 1.1	10.0 ± 0.7
Lactic	718.5 ± 3.8	1,417.1 ± 0.3	1,735.0 ± 9.7	2,465.8 ±25.9	2,259.4 ±83.1	2,424.5 ± 9.5
Uric	23.1 ± 0.7	7.4 ± 0.1	19.5 ± 0.1	3.7 ± 0.1	2.4 ± 0.1	1.8 ± 0.0
Acetic	283.3 ±13.0	223.2 ±14.5	401.5 ±14.4	250.5 ±12.0	260.2 ± 8.9	250.5 ± 1.1
Propionic	16.7 ± 0.2	11.7 ± 1.1	14.3 ± 0.3	11.9 ± 0.2	11.9 ± 0.1	11.8 ± 0.1

(cont.)

Table 4.14 (cont.). Organic acids (ppm) present at various stages of Cheddar cheese production (batch B).

	Ripening stage (days)						
Acid	19	43	69	92	118	133	174
Citric	22.1 ± 0.6	20.3 ± 1.5	19.5 ± 2.7	25.1 ± 4.0	22.9 ± 2.1	17.9 ± 2.2	18.4 ± 1.8
Orotic	7.8 ± 0.4	7.8 ± 0.2	6.6 ± 0.9	8.2 ± 0.2	5.5 ± 0.2	8.6 ± 0.2	7.1 ± 0.8
Pyruvic	9.1 ± 0.9	11.2 ± 0.0	14.0 ± 3.8	12.5 ± 0.7	11.7 ± 2.4	8.1 ± 1.1	8.3 ± 0.5
Lactic	3,964.5 ± 5.4	4,150.4 ± 9.7	3,828.5 ±27.3	4,227.3 ±23.8	4,407.1 ± 2.1	4,388.1 ±46.8	4,417.6 ±21.1
Uric	2.3 ± 0.0	4.3 ± 0.1	1.5 ± 0.0	1.3 ± 0.1	0.7 ± 0.2	1.2 ± 0.1	1.1 ± 0.1
Acetic	288.9 ± 8.6	266.2 ± 3.2	258.2 ±15.8	272.7 ± 5.7	258.7 ±17.6	296.0 ± 1.3	314.0 ±20.1
Propionic	15.8 ± 0.2	17.5 ± 0.4	18.3 ± 0.4	16.6 ± 0.8	15.7 ± 0.7	15.8 ± 0.1	16.8 ± 0.7

under the same conditions. Both batches showed the same trend in acid production; the differences were due to small fluctuations in butterfat content in the milk and fluctuations in temperatures. The results did not reveal major differences in the organic acids of the two batches.

The most abundant acid in Cheddar cheese was lactic acid, which increased steadily during the process and reached 4,430.6 ppm (in batch A) after 174 days of ripening. The increase was most rapid during the initial processing stages, and slower during the subsequent ripening period, especially at the whey drainage step. The amount of lactic acid in cheddar, batch B, was 4,417.6 ppm, 0.29% lower than batch A.

The next most abundant organic acid in Cheddar cheese was citric acid, which decreased from 878.4 ppm in batch A raw milk to 20.8 ppm in batch A end-product, while batch B end-product contained 18.4 ppm, 11.5% lower than batch A. The orotic acid content decreased from 72.2 ppm in raw milk to 8.8 ppm in the end-product of batch A, while batch B end-product contained only 7.1 ppm of orotic acid, 19% lower than in batch A. Pyruvic acid content decreased by 59% in the batch A process and by 57% in the batch B process. Batch A Cheddar contained 18% more pyruvic acid than batch B. Like all other organic acids, uric acid was retained mostly by the whey, so its content in the end product was low (0.4 ppm in batch A and 1.1 ppm in batch B). The concentration of uric acid in batch B was much higher than in batch A. Nevertheless, the amounts were so low that the difference

should be considered negligible. Acetic and propionic acids were not present in the raw milk, but increased gradually after culture addition. Batch A contained 290.4 ppm acetic acid, 7.5% less than batch B (314.0 ppm), and 188.2 ppm propionic acid, 12% more than batch B (168.0 ppm).

Marsili *et al.* (1981) detected in Cheddar similar amounts of citric (25 ppm) and orotic acid (4.9 ppm). However, they found higher levels of all other organic acids present, and in addition 420 ppm of formic acid, while our results did not confirm its presence. The data are difficult to compare, because Marsili *et al.* (1981) give no information on the age or processing procedure of their sharp Cheddar cheese.

4.5.1.4 Mozzarella (pressed and stretched)

Table 4.15 contains results for pressed Mozzarella, which resembles Cheddar cheese and was made with a similar bacterial culture (*Streptococcus lactis* and *S. cremoris*). Stretched Mozzarella (Table 4.16), on the other hand, was produced by the addition of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* to the lactic acid-producing bacteria (*S. lactis* and *S. cremoris*). It also included a second cooking stage at 73°C, where the curd was simultaneously stretched. In both cheeses, lactic acid was the most abundant acid present, but the concentration was much higher in the pressed cheese, which contained 4,165.8 ppm (stretched Mozzarella contained only 2,552.2 ppm).

From Table 4.15, it can be seen that propionic acid was present in high quantities (70.0 ppm) in pressed Mozzarella

Table 4.15. Organic acids (ppm) present at various stages of pressed Mozzarella cheese production.

Acid	Milk		Addition of			Cutting
	raw	pasteurized	$CaCl_2$	culture	rennet	
Citric	1,000.2 ±21.3	1,026.8 ± 9.2	1,050.5 ±33.3	1,078.3 ±41.2	1,027.1 ±16.4	1,023.8 ± 1.6
Orotic	86.6 ± 2.9	91.3 ± 1.1	89.7 ± 0.7	87.9 ± 0.6	88.8 ± 3.9	89.5 ± 0.2
Pyruvic	21.9 ± 1.2	23.7 ± 0.5	23.2 ± 0.9	23.6 ± 0.5	21.8 ± 0.4	22.4 ± 0.2
Lactic	<60	<60	200.6 ±13.2	230.5 ±15.5	272.3 ± 4.8	305.3 ±24.2
Uric	26.8 ± 0.2	26.7 ± 0.2	27.2 ± 0.0	27.2 ± 0.1	26.5 ± 0.5	32.9 ± 0.0
Acetic	n.d.	n.d.	n.d.	n.d.	38.4 ± 3.2	50.9 ± 1.6
Propionic	n.d.	n.d.	n.d.	10.6 ± 0.3	10.8 ± 0.6	11.0 ± 0.5

(cont.)

Table 4.15 (cont.). Organic acids (ppm) present at various stages of pressed Mozzarella cheese production.

Acid	Cooking	Whey drainage	Curd washing	Presalted curd	Before 2nd salting
Citric	971.9 ± 3.4	1,512.0 ±36.5	48.5 ± 0.3	33.0 ± 2.3	29.7 ± 1.5
Orotic	89.4 ± 3.5	95.2 ± 0.7	29.9 ± 0.2	21.9 ± 0.3	12.4 ± 0.0
Pyruvic	20.0 ± 2.6	17.3 ± 4.2	12.3 ± 2.1	13.1 ± 1.3	13.4 ± 0.6
Lactic	352.1 ± 1.4	393.3 ± 5.6	844.7 ±30.7	1,070.4 ±11.2	1,474.5 ±12.8
Uric	32.8 ± 0.9	33.0 ± 3.0	14.5 ± 0.2	12.9 ± 0.1	10.6 ± 0.0
Acetic	56.9 ± 2.0	62.9 ± 0.1	51.7 ± 1.2	52.4 ± 0.7	55.1 ± 0.9
Propionic	16.7 ± 1.3	48.8 ± 0.3	28.8 ± 0.7	46.0 ± 0.8	47.1 ± 0.8

(cont.)

Table 4.15 (cont.). Organic acids (ppm) present at various stages of pressed Mozzarella cheese production.

Acid	Moulding	Finished product	Ripening stage (days)		
			13	22	30
Citric	34.8 ± 0.8	24.6 ± 2.2	30.2 ± 2.4	24.1 ± 2.4	29.5 ± 3.7
Orotic	12.8 ± 0.8	6.5 ± 0.0	18.5 ±14.8	3.7 ± 0.2	2.2 ± 0.1
Pyruvic	11.4 ± 1.5	12.0 ± 0.9	13.8 ± 1.1	14.6 ± 0.3	15.9 ± 1.0
Lactic	1,217.3 ±36.2	3,965.3 ±10.9	4,090.0 ±39.3	4,135.3 ±17.2	4,165.8 ±14.5
Uric	9.1 ± 0.2	6.0 ± 0.1	6.2 ± 0.1	5.7 ± 0.1	5.8 ± 0.1
Acetic	55.5 ± 2.4	63.9 ± 6.5	63.0 ± 3.8	69.5 ± 7.6	61.0 ± 5.1
Propionic	47.7 ± 1.0	51.5 ± 0.6	61.0 ± 0.3	67.2 ± 1.5	70.0 ± 0.9

Table 4.16. Organic acids (ppm) present at various stages of stretched Mozzarella cheese production.

Acid	Raw milk	Pasteurized milk and $CaCl_2$ added	Addition of		Cutting	Stirring
			culture	rennet		
Citric	876.1 ± 0.3	886.5 ± 1.9	876.7 ± 4.1	860.6 ±29.6	915.2 ± 2.8	956.5 ±10.3
Orotic	79.9 ± 0.9	82.3 ± 0.1	80.0 ± 0.2	81.0 ± 0.8	83.5 ± 0.4	82.8 ± 0.3
Pyruvic	24.9 ± 0.3	20.9 ± 0.4	21.3 ± 1.0	21.7 ± 0.4	23.7 ± 0.1	23.4 ± 0.2
Lactic	<60	<60	218.4 ±16.5	223.7 ± 2.3	298.3 ±11.9	311.0 ± 1.6
Uric	26.2 ± 0.3	25.7 ± 0.2	26.3 ± 0.0	23.9 ± 0.3	22.8 ± 0.0	23.5 ± 0.3
Acetic	n.d.	n.d.	n.d.	n.d.	n.d.	24.8 ± 0.8
Propionic	n.d.	n.d.	n.d.	n.d.	n.d.	2.7 ± 0.1

(cont.)

Table 4.16 (cont.). Organic acids (ppm) present at various stages of stretched Mozzarella cheese production.

Acid	Cooking	Whey after cooking	Curd after cooking	Whey at drainage	Dry curd after drainage	Dry curd 1 hr later, before cooking
Citric	905.2 ± 3.6	1,156.8 ±40.3	243.1 ± 6.2	1,146.2 ± 8.1	52.3 ± 6.4	53.9 ± 0.3
Orotic	78.8 ± 0.8	83.5 ± 1.1	53.6 ± 0.2	83.8 ± 0.8	35.5 ± 0.1	14.0 ± 0.1
Pyruvic	25.7 ± 0.1	26.2 ± 0.4	27.1 ± 0.1	24.5 ± 0.3	33.9 ± 0.5	35.0 ± 1.7
Lactic	435.3 ± 3.0	389.0 ± 2.9	581.6 ± 1.3	444.2 ± 1.1	1,434.6 ±10.2	3,335.3 ± 3.0
Uric	24.2 ± 0.1	29.5 ± 0.1	15.3 ± 0.2	27.5 ± 0.4	13.9 ± 0.0	10.9 ± 0.1
Acetic	50.0 ± 2.5	94.8 ± 3.4	97.7 ± 1.0	131.4 ± 0.4	98.6 ± 7.0	111.6 ± 6.2
Propionic	3.5 ± 0.1	3.9 ± 0.2	3.3 ± 0.2	5.5 ± 0.3	5.3 ± 0.3	7.9 ± 0.1

(cont.)

Table 4.16 (cont.). Organic acids (ppm) present at various stages of stretched Mozzarella cheese production.

Acid	Dry moulded curd	Finished cheese, next day	Ripening stage (days)		
			4	12	17
Citric	50.5 ± 3.8	55.1 ± 4.0	51.7 ± 2.1	55.0 ± 3.2	51.8 ± 0.5
Orotic	11.8 ± 1.0	10.1 ± 0.1	10.9 ± 0.1	12.2 ± 0.3	13.7 ± 0.0
Pyruvic	27.2 ± 0.2	25.7 ± 0.0	36.1 ± 2.1	36.5 ± 1.9	40.0 ± 0.9
Lactic	2,785.1 ± 6.5	2,507.8 ± 5.8	2,577.7 ±20.0	2,565.4 ±13.7	2,552.5 ± 6.8
Uric	10.2 ± 0.3	12.5 ± 2.0	12.2 ± 0.3	9.1 ± 0.1	9.7 ± 0.1
Acetic	111.8 ± 2.3	114.8 ± 2.0	112.9 ± 3.9	104.0 ± 1.2	105.4 ± 6.1
Propionic	8.6 ± 0.2	8.3 ± 0.2	14.5 ± 1.0	18.7 ± 2.7	19.7 ± 0.9

after 30 days of ripening, similar to acetic acid, which was 61.0 ppm. Both acetic and propionic acids were detected only after culture addition and increased during processing stages and subsequent ripening of the cheese. Citric acid decreased significantly, from 1,000.2 ppm in raw milk to 29.5 ppm in the finished cheese. The orotic acid concentration also decreased greatly; from 86.6 ppm to 2.2 ppm (a decrease of 97.5%). Pyruvic acid content was reduced from 21.9 ppm in raw milk to 15.9 ppm in the finished cheese, i.e., by 27.4%. Uric acid decreased from 26.8 ppm to 5.8 ppm, a decrease of 78.4%. It appears that these acids were utilized by microorganisms in their metabolic pathways.

Table 4.16 shows that the same trend of acid production and consumption was followed in stretched Mozzarella cheese. Pyruvic acid was an exception: it increased during production of stretched Mozzarella, contrary to the decrease found in pressed Mozzarella production. The stretched cheese contained, in ppm, 51.8 citric , 13.7 orotic, 40.0 pyruvic and 9.7 uric acids; all these values were much lower in pressed Mozzarella. Stretched cheese contained a high amount of acetic acid (105.4 ppm), which was detected first during the stirring stage, i.e., after curd setting. Propionic acid was also first detected at the same processing stage, acquiring a concentration of 8.3 ppm on the following day. During cheese ripening this value increased to 19.7 ppm in the finished product. In conclusion, in terms of both organic acid profiles and bacterial culture added, these two Mozzarella cheeses were indeed very different.

4.5.1.5 Quark

Quark is a lactic curd cheese, where the whey is separated from the curd at the end of a 9-hr incubation period. Table 4.17 shows that lactic acid is the dominant acid. During processing, lactic acid increased from under 60 ppm in the raw milk to 3,349.9 in the end product and 5,836.3 in the whey. This increase was rapid after the first 40 min of incubation to about 8 hr, where it started slowing down (see Figure 4.5). Similar to cottage cheese, Quark cheese was unripened, but its lactic acid content was much higher.

Acetic acid was present at very high levels both in the cheese (443.5 ppm) and whey (623.0 ppm). It increased sharply after culture addition, and the level was 66.2 ppm after 40 min of incubation. Citric acid decreased from 943.0 ppm in the raw milk to 176.2 ppm in Quark cheese; while orotic acid decreased from 72.6 ppm to 22.7 ppm. Pyruvic acid increased during the incubation period, but was transferred predominantly to the whey fraction (50.4 ppm); only 12.7 ppm were found in the cheese. Uric acid was found to be 10.6 ppm in Quark and 16.7 ppm in whey; while propionic acid was 13.6 ppm in cheese and 18.0 ppm in whey. In conclusion, it appeared that all acid contents were relatively high in Quark, due to the high content of whey retained by this cheese.

4.5.1.6 Ricotta

Ricotta curd was produced by high temperature and acid precipitation of protein. In production of this cheese the

Table 4.17. Organic acids (ppm) present at various stages of Quark production.

Acid	Raw milk	Pasteurized milk	Culture addition	Incubation time		
				40 min	1 hr (rennet addition)	2 hr
Citric	943.0 ±15.1	886.0 ±10.9	944.9 ±42.2	951.2 ±47.7	913.7 ±34.3	881.6 ± 2.0
Orotic	72.6 ± 0.1	71.7 ± 0.8	73.2 ± 0.7	74.0 ± 1.4	72.6 ± 0.3	72.1 ± 0.2
Pyruvic	22.5 ± 0.6	26.0 ± 0.8	20.8 ± 1.5	21.4 ± 1.7	22.0 ± 3.3	31.7 ± 1.4
Lactic	<60	<60	284.6 ± 2.8	315.1 ± 2.3	371.3 ± 4.8	484.7 ± 2.2
Uric	16.7 ± 0.1	16.8 ± 0.1	18.2 ± 0.2	16.6 ± 0.0	14.8 ± 0.0	20.8 ± 0.1
Acetic	n.d.	n.d.	n.d.	66.2 ± 1.2	82.9 ± 0.4	88.1 ± 3.6
Propionic	n.d.	n.d.	6.6 ± 0.1	7.0 ± 0.3	7.6 ± 0.1	8.3 ± 0.1

(cont.)

Table 4.17 (cont.). Organic acids (ppm) present at various stages of Quark production.

Acid	Incubation time (hr)				Finished product (next day)	Whey (next day)
	3.5	5.75	7.75	9		
Citric	788.2 ± 3.9	761.1 ±17.5	759.0 ± 2.1	750.2 ± 5.8	176.2 ± 1.9	383.4 ± 0.5
Orotic	71.3 ± 0.0	62.3 ± 0.6	46.0 ± 0.5	43.0 ± 0.7	22.7 ± 0.8	44.5 ± 0.0
Pyruvic	25.7 ± 0.2	26.6 ± 0.4	35.1 ± 0.1	33.3 ± 1.2	12.7 ± 1.3	50.4 ± 0.9
Lactic	590.6 ± 7.6	1,661.5 ±13.2	3,726.4 ±46.9	4,225.7 ±43.6	3,349.9 ±13.1	5,836.3 ±87.5
Uric	21.2 ± 0.0	21.5 ± 0.0	19.2 ± 0.2	16.4 ± 0.1	10.6 ± 0.1	16.7 ± 0.1
Acetic	114.2 ± 3.5	134.1 ± 3.0	204.5 ± 6.2	302.9 ±10.7	443.5 ± 2.5	623.0 ±11.3
Propionic	9.8 ± 0.3	11.1 ± 0.1	11.6 ± 0.2	12.5 ± 0.3	13.6 ± 1.4	18.0 ± 2.0

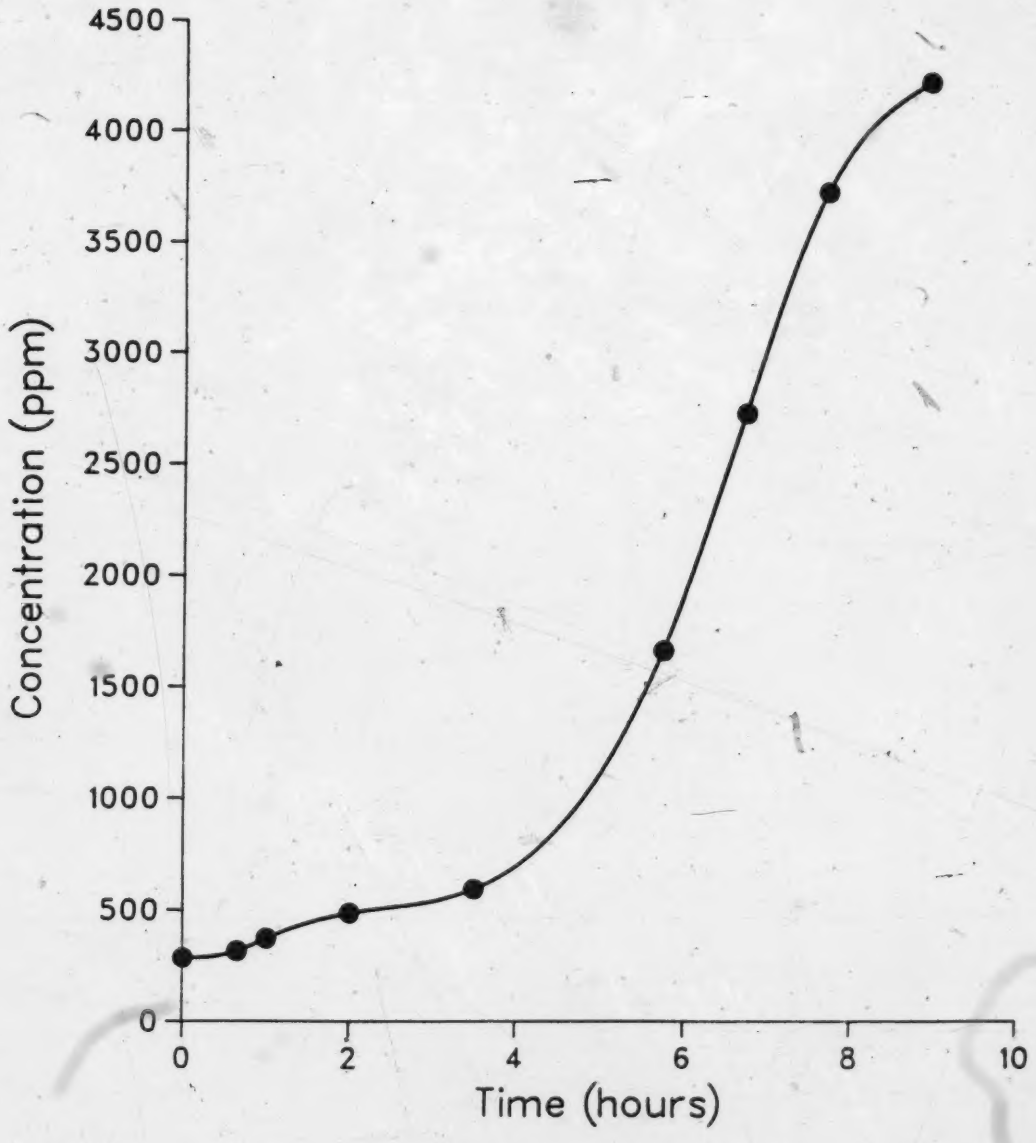

Figure 4.5 Lactic acid formation (ppm) during incubation of milk in the production of Quark.

source of protein was the casein from added milk and whey proteins from Mozzarella and Ricotta whey collected from a previous batch. The acid precipitation was achieved by lactic acid in Ricotta whey plus added citric acid, and also by the citric acid present in Mozzarella whey. Table 4.18 shows an organic acid profile very different from other cheeses. The major source of acids was the citric acid-enriched Ricotta whey, and less so the other components used in the process.

The end product contained 515.8 ppm of lactic acid, which is close to the value found for cottage cheese. Lactic acid content was low as no bacterial culture was added to the whey and cream used in the process. Citric acid was the second most abundant acid at 294.1 ppm, similar again to cottage cheese. Orotic acid content, on the other hand, was higher: 56.3 ppm. Pyruvic acid concentration was 12.8 ppm and that of uric acid 17.0 ppm. A high amount of acetic acid was present (129.7 ppm), along with low amounts of propionic and hippuric acids (10.7 ppm and and 8.7 ppm, respectively). The high level of acetic acid was probably due to the presence of spoilage microorganisms or to the added acetic acid not declared in process formulation.

4.5.2 Imported cheeses

4.5.2.1 Cheeses from Denmark

Danish Camembert (Table 4.19) showed a different organic acid profile from domestic Camembert (Table 4.11). Lactic acid was present in a much lower amount (765.4 ppm),

Table 4.18. Organic acids (ppm) present at various stages of Ricotta cheese production.

Acid	Acidified Ricotta whey	Pressed Mozzarella whey	Cooked Mozzarella whey after salting	Addition of milk and cream	Addition of acidified Ricotta whey	Finished product
Citric	1,911.9 ±10.2	1,536.3 ±19.7	1,455.6 ± 3.2	1,047.6 ±12.1	782.9 ±10.2	294.1 ±22.3
Orotic	4.2 ± 0.2	95.5 ± 0.5	94.1 ± 0.9	82.6 ± 0.2	77.8 ± 0.6	56.3 ± 0.7
Pyruvic	198.1 ± 0.7	14.9 ± 0.7	15.1 ± 3.1	15.1 ± 1.3	17.8 ± 1.6	12.8 ± 0.3
Lactic	7,853.9 ± 9.8	439.7 ±31.8	444.3 ±15.5	348.6 ± 1.3	545.8 ± 6.5	515.8 ± 9.0
Uric	32.1 ± 0.0	32.3 ± 0.2	31.7 ± 0.3	27.2 ± 0.2	27.3 ± 0.1	17.0 ± 0.1
Acetic	382.2 ± 8.7	73.7 ± 8.9	114.8 ± 6.1	n.d.	159.0 ± 8.1	129.7 ± 5.8
Propionic	166.1 ± 6.4	87.6 ± 0.5	58.1 ± 1.9	n.d.	79.2 ± 1.2	10.7 ± 0.2
Hippuric	---	---	---	---	---	8.7 ± 0.8

Table 4.19. Organic acids (ppm) present in cheeses imported from Denmark by the Canadian market.

Acid	Camembert	Danablue	Feta	Havarti	Samsoe
Citric	n.d.	n.d.	46.5 ± 1.6	n.d.	n.d.
Orotic	3.6 ± 0.1	1.5 ± 0.1	2.1 ± 0.2	1.3 ± 0.1	2.4 ± 0.0
Pyruvic	18.0 ± 1.2	32.0 ± 1.7	32.5 ± 2.4	72.8 ± 0.8	16.6 ± 0.1
Lactic	765.4 ±13.7	2,406.6 ±46.3	3,268.4 ±17.0	1,599.2 ±13.7	1,368.2 ± 5.3
Uric	n.d.	16.2 ± 0.1	n.d.	n.d.	n.d.
Formic	209.0 ± 6.0	244.8 ± 4.1	94.9 ±12.1	560.3 ±40.8	n.d.
Acetic	24.0 ± 2.1	250.7 ±15.6	168.0 ± 2.7	1113.5 ±12.6	795.1 ± 7.6
Propionic	88.2 ± 0.3	570.2 ± 7.0	34.6 ± 0.7	160.0 ± 4.1	298.5 ± 0.9
Butyric	---	<450	---	---	---
Hippuric	18.4 ± 0.9	n.d.	22.7 ± 1.0	---	14.7 ± 0.3

while a higher amount of formic acid was detected (209.0 ppm). Citric and uric acids were not detectable, while orotic and pyruvic acids were 3.6 ppm and 18.0 ppm, respectively. Acetic acid was 24.0 ppm, which is much lower than in the Canadian cheese; while propionic acid was 88.2 ppm, a content much higher than in the Canadian Camembert. Also, the presence of hippuric acid, at 18.4 ppm, was detected.

Danablue is produced by the addition of *Penicillium candidum* and *P. roqueforti* mould spores to the cheese curd, and stabbing the cheese for their growth in the presence of oxygen. The cheese (Table 4.19) contained, in order of concentration, lactic (2,406.6 ppm), acetic (657.7 ppm), propionic (570.2 ppm), formic (244.8 ppm), pyruvic (32.0 ppm) and uric acids (16.2 ppm). Citric and hippuric acids were not detected.

Danish Feta cheese (Table 4.19) is a white brined cheese, with a short period of ripening. Its moisture content is 52.0%, so the acid content is higher than in cheeses with a lower moisture content. The most abundant acid present is again lactic acid (3,268.4 ppm), followed by acetic (168.0 ppm) and formic acids (94.9 ppm). Citric acid (46.5 ppm) is relatively high in comparison to other cheeses. Propionic (34.6 ppm) and pyruvic acids (32.5 ppm) were very close to those of Danablue. Hippuric acid was also present (22.7 ppm).

Danish Havarti and Samsoe are cheeses with holes, which are the result of addition of propionic acid bacteria in the

cheese process. Their major acid (Table 4.19) was lactic acid (1,599.2 ppm in Havarti; and 1,368.2 ppm in Samsoe). Other acids of high concentration included acetic (1,113.5 ppm and 795.1 ppm) and propionic acids (160.0 ppm and 298.5 ppm). Formic acid was not present in Samsoe, but was very high (560.3 ppm) in Havarti. Pyruvic acid was also much higher in Havarti (72.8 ppm) than in Samsoe (16.6 ppm). Orotic acid was low in both Havarti (1.3 ppm) and Samsoe (2.4 ppm); and citric and uric acids could not be detected in either product. Hippuric acid was present only in Samsoe (14.7 ppm).

4.5.2.2 Cheeses from France

French Brie, Camembert and Marcillat are produced with the addition of *Penicillium candidum* mould, while Roquefort has *Penicillium roqueforti* spores added. Marcillat was produced from goat milk, while Roquefort cheese was made from non-pasteurized whole ewe's milk. Port Salut, on the other hand, a semi-hard cheese, was made from cow's milk, without any mould addition. The differences in the composition of milks from different animals can be seen in Table 4.20a.

Table 4.20a. Composition (%) of Cow, Goat and Sheep Milk (according to Veisseyre, 1979).

Substance	Cow	Goat	Sheep
Fat	3.5-4.0	3.5-5.5	5.5-7.0
Lactose	4.7-5.2	4.0-5.0	4.3-5.0
Casein	2.7-3.0	3.0-3.2	4.5-5.0
Albumin and Globulin	0.4-0.5	0.5-0.7	0.8-1.0
Ash	0.9-0.95	0.7-0.9	0.9-1.0

For all three of the white mould cheeses, the most abundant organic acid was lactic: 1,535.7 ppm in Camembert; 1204.8 ppm in Marcillat; and 1107.2 ppm in Brie (Table 4.20). The second most abundant acid in Brie was formic (520.4 ppm), followed by acetic acid (141.8 ppm). Camembert contained less formic acid (427.0 ppm), and 116.6 ppm acetic acid. Marcillat contained 146.0 ppm formic acid, and did not contain acetic or hippuric acids. Propionic acid ranged from 68.3 ppm in Camembert to 32.2 ppm in Marcillat. Uric acid was detected only in Brie (4.4 ppm), and orotic acid ranged from 1.7 ppm in Camembert to 20.4 ppm in Marcillat. Citric acid was not detected in any of the French cheeses analyzed.

Port Salut acid composition, also shown in Table 4.20, showed high amounts of lactic (2,828.3 ppm), formic (1,651.9 ppm), acetic (751.7 ppm) and propionic acids (528.4 ppm). Citric and uric acids could not be detected, and the amounts of orotic and hippuric acids were low (1.5 and 3.9 ppm, respectively).

Lastly, Roquefort contained much lower lactic acid than the other French cheeses (497.7 ppm), but had high content

Table 4.20. Organic acids (ppm) present in cheeses imported from France by the Canadian market.

Acid	Brie	Camembert	Marcillat	Port Salut	Roquefort
Citric	n.d.	n.d.	n.d.	n.d.	n.d.
Orotic	9.7 ± 0.4	1.7 ± 0.1	20.4 ± 0.2	1.5 ± 0.1	1.6 ± 0.4
Pyruvic	49.3 ± 0.6	17.4 ± 0.3	19.7 ± 0.1	31.4 ± 0.5	41.3 ± 0.1
Lactic	1,107.2 ± 0.9	1,535.7 ± 0.2	1,204.8 ± 8.3	2,828.3 ±22.0	497.4 ± 6.7
Uric	4.4 ± 0.0	n.d.	n.d.	n.d.	n.d.
Formic	520.4 ± 3.7	427.0 ± 2.2	146.0 ± 2.5	1,651.9 ±12.1	66.5 ±15.9
Acetic	141.8 ± 1.4	116.6 ± 0.2	n.d.	751.7 ±39.3	71.3 ± 2.7
Propionic	35.1 ± 4.7	68.3 ± 2.3	32.2 ± 0.1	119.2 ± 6.5	528.4 ± 9.1
Butyric	---	---	---	---	<450
Hippuric	10.9 ± 0.6	8.4 ± 0.3	---.	3.9 ± 0.8	n.d.

(cont.)

of propionic acid (528.4 ppm). Formic and acetic acids were 66.5 ppm and 71.3 ppm, respectively; while pyruvic acid was 41.3 ppm. Orotic acid was 1.6 ppm, while citric, uric and hippuric acid contents were not detectable.

Larson and Hegarty (1979) reported 15 ppm of orotic acid in Roquefort cheese, while for blue cheese, Marsili *et al.* (1981) found 7.3 ppm of orotic. Ashoor and Wetty (1984) found 12,750 ppm of lactic acid in blue cheese, but Marsili *et al.* (1981) reported only 3,080 ppm. The latter authors also found the following concentrations of acids in blue cheese: 40 ppm citric, 27 ppm pyruvic, 20.8 ppm uric, 420 ppm formic and 250 ppm of acetic acid. Hippuric acid content was under 2 ppm.

4.5.2.3 Cheeses from the Netherlands

Edam and Gouda differ in their texture. Edam cheese has a close texture, while Gouda contains a few eye holes. As seen in Table 4.21, both cheeses contained a high content of lactic acid: 2,032.4 ppm in Edam and 1,894.2 ppm in Gouda. Acetic acid was also in high concentration, 1,294.3 and 1,046.1 ppm, respectively. In addition, Edam contained 663.6 ppm formic acid, while Gouda contained 231.0 ppm propionic acid.

4.5.2.4 Cheeses from Switzerland

All three cheeses listed in Table 4.21 had propionic acid bacteria added to the lactic acid culture. This accounts from the high propionic acid content found in

Table 4.21. Organic acids (ppm) present in cheeses imported from the Netherlands and Switzerland by the Canadian market.

	Dutch		Swiss		
Acid	Edam	Gouda	Appensell	Emmental	Gruyère
Citric	n.d.	n.d.	n.d.	n.d.	n.d.
Orotic	0.7 ± 0.1	0.8 ± 0.1	n.d.	4.3 ± 0.2	0.9 ± 0.1
Pyruvic	42.0 ± 0.8	62.7 ± 2.0	8.1 ± 1.4	43.4 ± 0.4	47.9 ± 0.4
Lactic	2,032.4 ±28.5	1,894.2 ±29.7	833.4 ± 5.0	150.4 ± 2.2	1,070.1 ±14.0
Uric	n.d.	n.d.	n.d.	n.d.	n.d.
Formic	66.3 ± 0.8	67.0 ± 0.8	409.7 ± 8.1	641.9 ± 8.7	499.5 ± 3.9
Acetic	1,294.3 ±11.5	1,046.1 ± 2.9	1,255.9 ±53.9	2,142.7 ±49.0	536.8 ± 7.4
Propionic	87.4 ± 0.3	231.0 ± 3.8	220.8 ± 1.0	874.6 ± 6.7	298.3 ± 4.9
Hippuric	---	7.9 ± 0.6	6.8 ± 0.4	5.8 ± 0.2	24.4 ± 1.2

Emmental cheese, 874.6 ppm. The amount of this acid in Gruyère cheese was 298.3 ppm and in Appenzell 220.8 ppm. The major organic acid in Emmental and Appenzell was acetic acid, 2,142.7 and 1,255.9 ppm, respectively. In Gruyère cheese this amount was only 536.8 ppm. The latter cheese contained 1,070.1 ppm lactic acid. This acid content was lower in Appenzell (833.4 ppm) and even lower in Emmental cheese (150.4 ppm). The lactic acid precursor, pyruvic acid, was 43.4-47.9 ppm in Emmental and Gruyère, and was only 8.1 ppm in Appenzell cheese.

Jager and Tschager (1983) reported over 1,244 ppm lactic acid in quality grade I Emmental, a propionic acid content under 267 ppm, and an acetic acid level of 255 ppm. Moreover, they reported the presence of succinic acid (43 ppm); this acid was also detected by us but it coeluted with polypeptides and hence its content was not reported. In addition, they found 59 ppm formic, 12 ppm citric and 2 ppm pyruvic acid. Also, the authors compared these results with two other cheeses of lower quality grades, and the difference in the organic acid profile was significant. The major difference was the decreased content of lactic acid, a doubling in the amount of succinic acid and an increase in propionic acid content of close to one-third in lower grade cheeses.

4.5.3 Statistical evaluation of the results

Statistical data related to the dependence of the organic acid content on the whey retained by the product are shown in Table 4.22. Positive correlation was found to be the highest in citric acid, i.e. its concentration increased with increase in moisture content in the product. The same can be applied for uric acid, which was positively but weakly correlated to the moisture content. Formic, acetic and propionic acids, on the other hand, had negative correlations with moisture content, which means that their concentrations decreased with moisture. The values for moisture contents of the dairy products analysed can be seen in Table 4.5. Table 4.23 presents the mean and standard deviation for organic acids in 5 replicates of pasteurized milk. The low values of standard deviations show that the LC method used was reproducible.

Table 4.22. Moisture content vs organic acid content correlation coefficients and probability values.

Acid	Correlation Coefficient (r)	Probability' (P)
Citric	0.5614	0.000
Orotic	0.2039	0.128
Pyruvic	-0.1871	0.149
Succinic	0.1840	0.153
Lactic	0.1763	0.163
Uric	0.2713	0.063
Formic	-0.2963	0.047
Acetic	-0.3168	0.036
Propionic	-0.3150	0.037
Hippuric	0.1325	0.231

' $P<0.05$; r is statistically significant.

Table 4.23. Reproducibility of the results of the liquid chromatographic method applied for the analysis of pasteurized milk.

Acid	Mean ($\bar{X}$)' (ppm)	Standard Deviation (SD)
Citric	1,120.94	3.50
Orotic	105.90	0.16
Pyruvic	31.99	0.66
Uric	14.46	0.02

' $\bar{X}$, mean of 5 repetitions.

5. CONCLUSION

Lactic acid was the most abundant organic acid in dairy products. Though its content was negligible in fluid and dehydrated milk and it could not be detected in creams, it was present in most fermented products. The content was highest in yogurt (7,955 ppm), followed by cultured buttermilk. Soft unripened cheeses like cottage and Quark also had a high content of lactic acid (about 4-6,000 ppm), followed by ripened cheeses with a range of 1,730-4,430 ppm. Imported cheeses, with the exception of Port Salut and Feta, the white brined cheese with a ripening period of less than one month, had a lactic acid range of 1,070-2,406 ppm. Emmental and Roquefort, the two cheeses characterized by a high content of propionic acid, had a lactic content less than 500 ppm. This agreed with a biochemical conversion of lactic into propionic acid known to occur in cheeses produced with propionic acid bacterial cultures. The lactic acid rate of formation was 6.4-6.5 ppm/min for cultured buttermilk, 11.7 and 13.6 ppm/min for Quark and cottage cheese, respectively, and was the highest in yogurt (32.2 ppm/min). All rates were calculated from the exponential phase of the incubation step of the process.

Lactic acid L- and D-isomers could not be separated by the LC-method applied. Hence, for comprehensive lactic acid determination the enzymatic method would still be indispensable.

Pyruvic acid, the intermediary product of bacterial metabolism, was found in all dairy products. For fluid milk, these results supported a possible use of the content of this acid as an index of bacterial quality.

Butyric acid was generally not detectable in the dairy products. Its content in fluid milk was less than 50 ppm, and in blue cheeses it was below 450 ppm.

In milk processing most of the organic acids were retained by whey; the acid content in curd merely reflected the extent of whey drainage. Moisture contents were correlated with individual organic acids analyzed. Highly significant correlation (at the 1% level) was obtained only for citric acid. This acid was not found in ripened cheeses, all of low moisture content. Formic, acetic and propionic acid contents were significantly correlated with the moisture content of the product. The correlation was found to be negative, which means that there was a decrease in acid concentration associated with increased moisture content. These acids were produced by bacterial culture added to the milk, and were found to be higher in dairy products with high butterfat and low moisture content. Uric acid was weakly correlated with moisture content (correlation coefficient of 0.27). This acid is known not to be synthesized by but is utilized by microorganisms, so it was expected to be present in higher amounts when a higher amount of whey was retained by the dairy product. The same pattern in correlation should be expected with orotic acid;

however, no correlation was found. On the other hand, pyruvic acid is an intermediary product in bacterial fermentations, so a correlation between its level and the amount of whey retained (moisture) would not be expected.

The standard deviation for 5 repetitions of the analysis of the organic acids present in pasteurized milk was 3.5 for citric acid, while orotic, pyruvic and uric acids had standard deviations below 1. Standard deviations for formic acid were not calculated: it would not reflect the precision of the analytical method since the acid was quantified by calculation. The other acids (lactic, acetic, propionic, butyric and hippuric) were either low in content or not detected in milk.

The applied LC-method was faster and simpler than conventional methods of analysis. Sample preparation involved addition of aqueous acetonitrile to the sample and centrifugation, to remove the precipitated protein, followed by injection of clarified supernatant into the LC-system. Sensitivity of the method ranged from 0.1-20.8 µg acid/ml and linear calibration curves were used over a wide concentration range.

In addition to 220 nm a wavelength of 275 nm was used for quantitation of uric acid and for revealing and correction for polypeptide interference which tended to inflate the results of several acids.

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7. APPENDIX -- LIQUID CHROMATOGRAMS OF ORGANIC ACIDS

The liquid chromatogram of the standard organic acids is presented in Figure 7.1, followed by the chromatograms of the organic acids in the various dairy products analyzed.

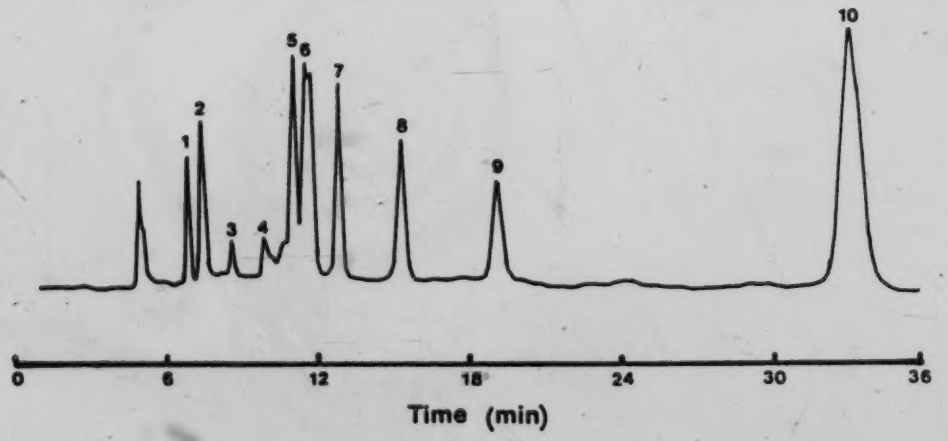

Figure 7.1. Liquid chromatogram of standard organic acids. Legend: (1) citric, 200 ppm; (2) orotic, 9 ppm; (3) pyruvic, 21 ppm; (4) succinic, 204 ppm; (5) lactic, 446.8 ppm; (6) uric, 6.3 ppm, and formic, 439 ppm; (7) acetic, 1049.2 ppm; (8) propionic, 992 ppm; (9) butyric, 750 ppm; (10) hippuric, 24 ppm.
The chromatographic conditions were as follows: 20 µl injections; 0.02 N H_2SO_4 as mobile phase; UV detector at 220 nm; column temperature 65°C; and flow rate 0.7 ml/min.
Sensitivity setting of the Bio-Rad detection system was 0.01 in Figure 7.1 and 0.02 in the following Figures.

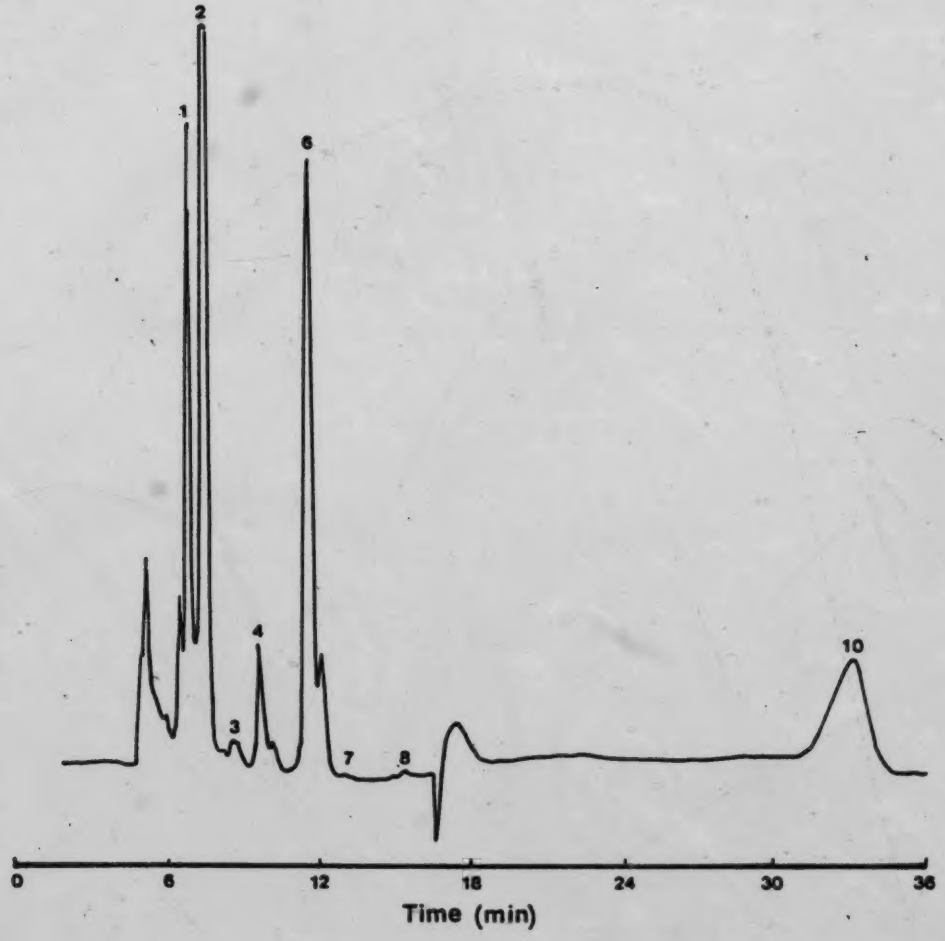

Figure 7.2. Liquid chromatogram of organic acids present in raw milk.
The milk sample was collected from NADP, Edmonton plant, after milk delivery from surrounding dairy farms by a bulk tank truck. BF content was 3.6%.
For peak identities in this and following figures, see Figure 7.1.

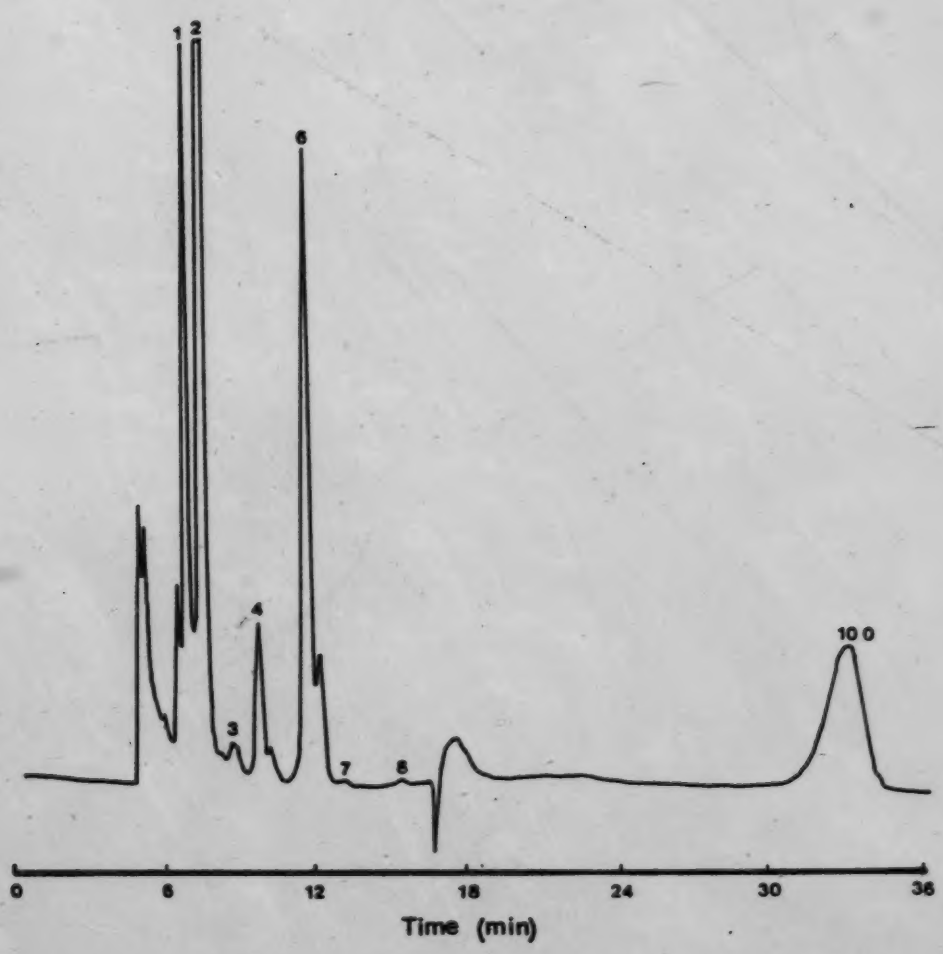

Figure 7.3. Liquid chromatogram of organic acids present in skim milk.
The skim milk was produced from raw milk by centrifugation, low pressure homogenization and HTST pasteurization in the Edmonton plant of NADP. BF content was 0.01-0.02%.

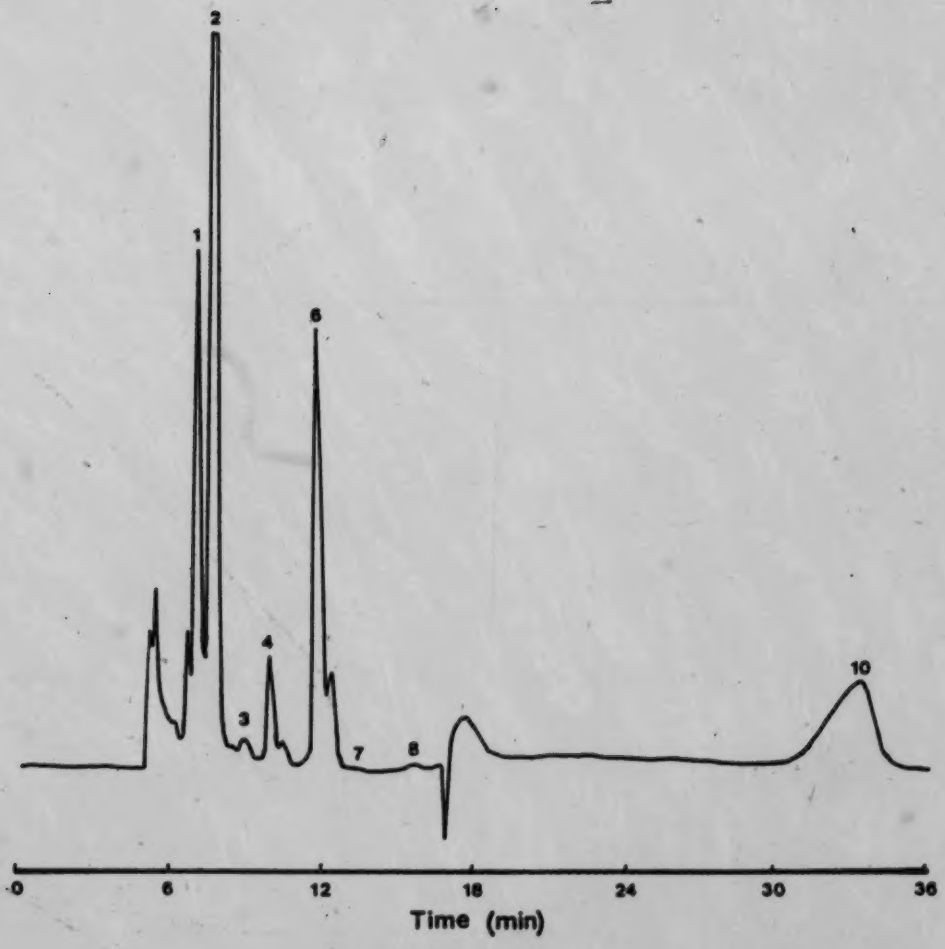

Figure 7.4. Liquid chromatogram of organic acids present in partly skimmed milk (2% BF).
The 2% BF milk was produced in the Edmonton plant of NADP from raw milk by its centrifugal separation into raw milk and cream, followed by blending to form 2% BF milk, and homogenization and pasteurization.

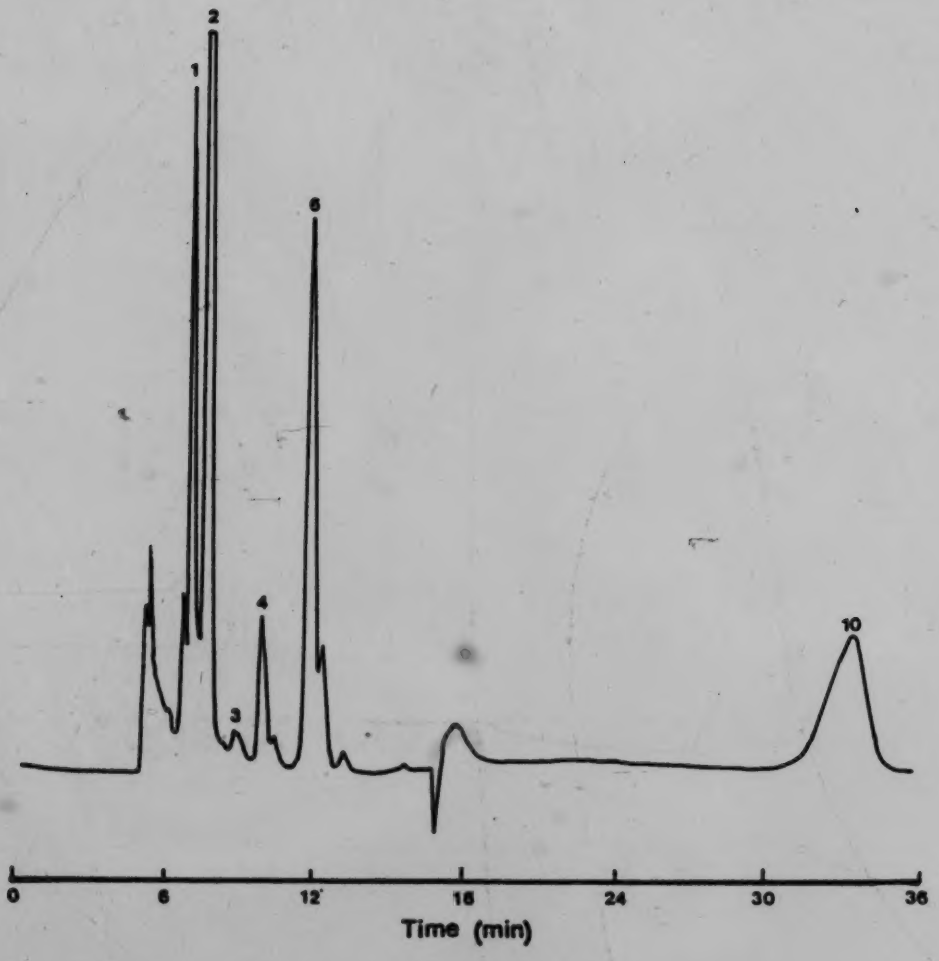

Figure 7.5. Liquid chromatogram of organic acids present in homogenized milk.
The homogenized milk was produced in the Edmonton plant of NADP from raw milk by a process similar to that given in Figure 6.4. BF content of homo milk was 3.25%.

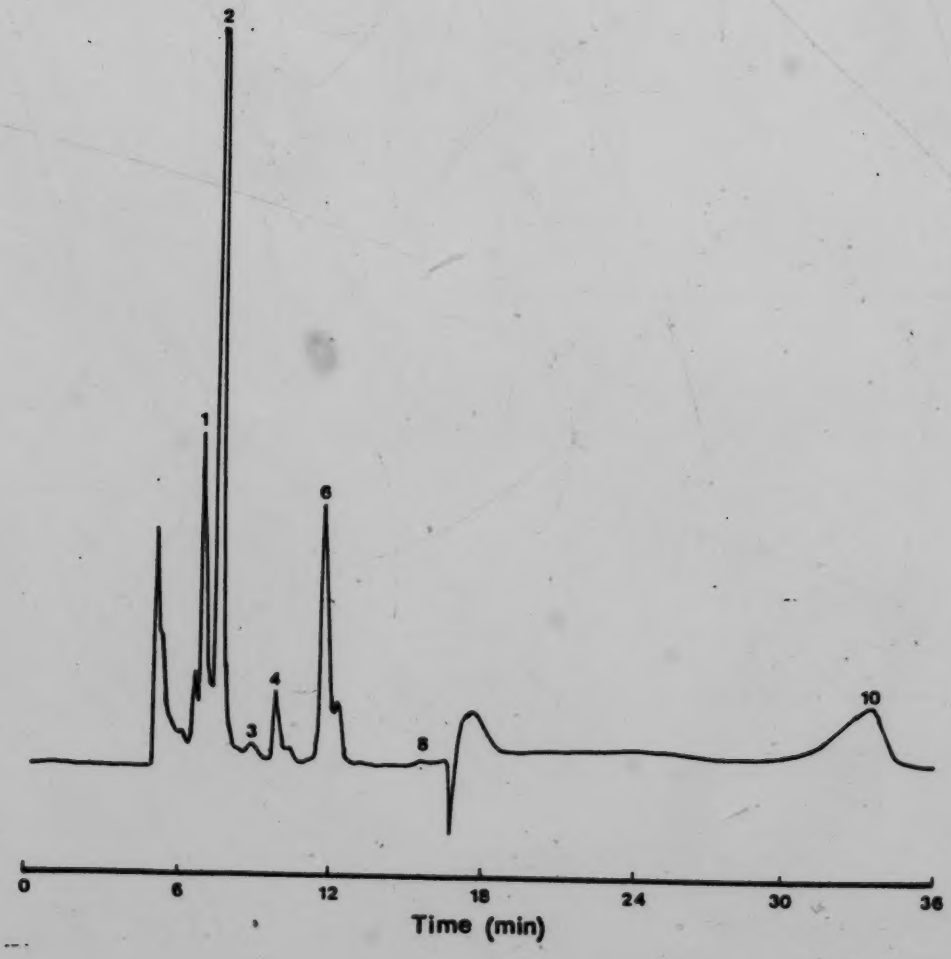

Figure 7.6. Liquid chromatogram of organic acids present in a commercial whipping cream.
Whipping cream was produced in the Edmonton plant of NADP by a process similar to that given in Figure 6.4. BF content was 32.5%.

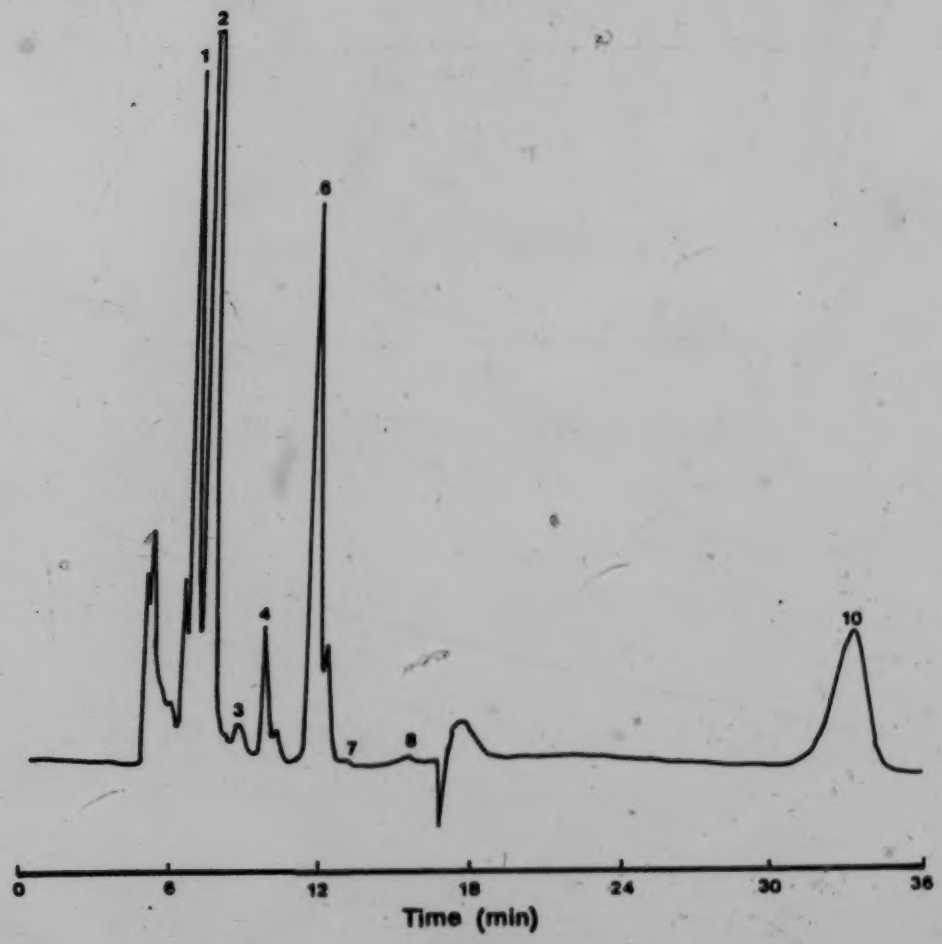

Figure 7.7. Liquid chromatogram of organic acids present in cereal cream.
Cereal cream was produced in the Edmonton plant of NADP from raw milk by a process similar to that given in Figure 6.4, except the homogenization step was omitted. BF content of cereal cream was 10.0%.

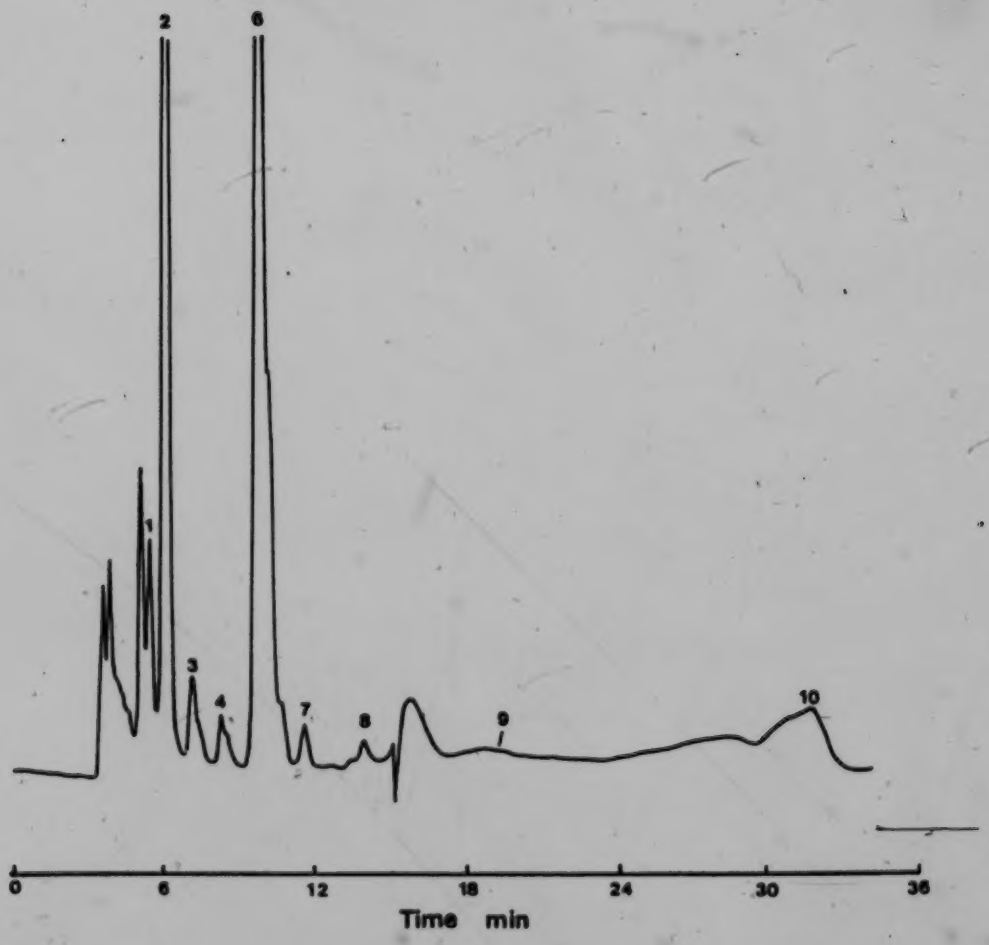

Figure 7.8. Liquid chromatogram of organic acids present in buttermilk powder.
Buttermilk powder was produced in the Barrhead plant of NADP from buttermilk, the liquid by-product of churning of cream to butter. The buttermilk was concentrated in a two-stage column vacuum evaporator and then spray dried in air at 95°C. The fat and moisture contents of the powder were 5.5 and 4.0%, respectively.

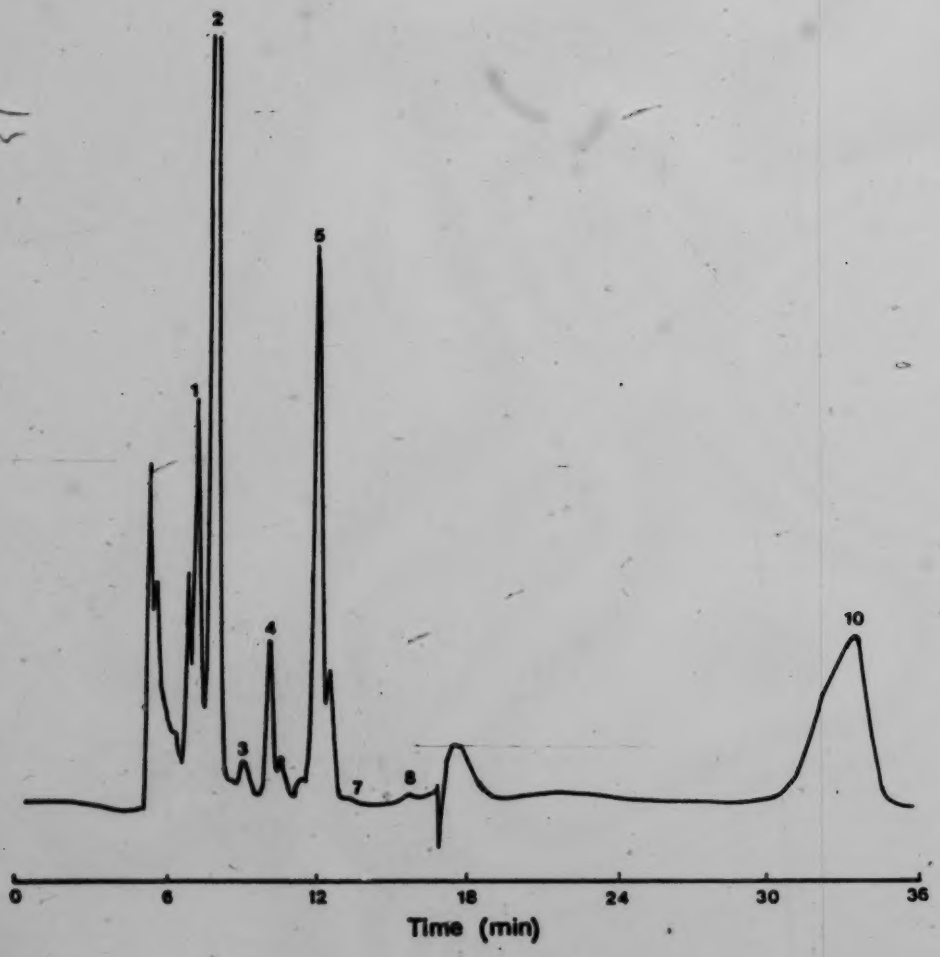

Figure 7.9. Liquid chromatogram of organic acids present in skim milk powder.
Skim milk powder was produced in the Barrhead plant of NADP from fluid skim milk (0.04% BF) by a preliminary vacuum evaporation followed by spray drying in air at 95°C. The fat and moisture contents of the product were 0.9 and 4.0%, respectively.

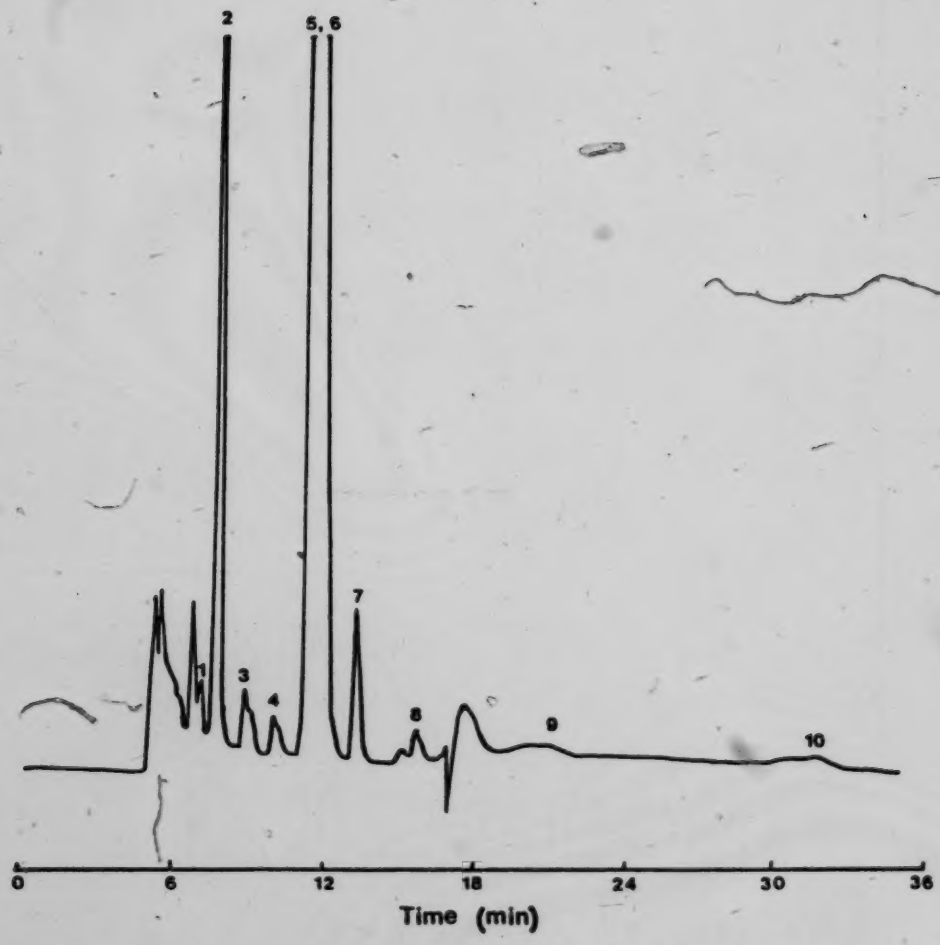

Figure 7.10. Liquid chromatogram of organic acids present in cultured buttermilk.
Cultured buttermilk was produced in the Edmonton plant of NADP. For production details see Experimental. The product contained 1.8% BF and 88.0% moisture.

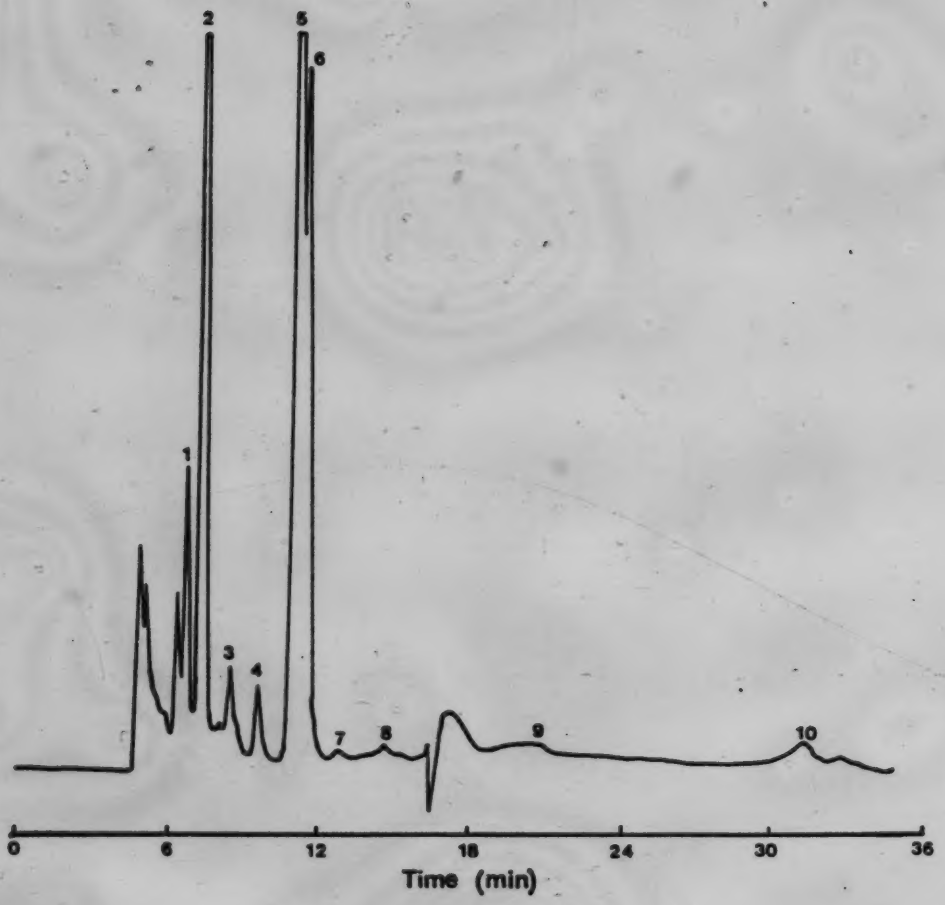

Figure 7.11. Liquid chromatogram of organic acids present in sour cream.
Sour cream was produced in the Edmonton plant of NADP from a blend of milk, cream and skimmed milk powder and stabilizers, which was inoculated with a lactic starter culture. For details see Experimental. The fat and moisture contents of the product were 15.5 and 86.3%, respectively.

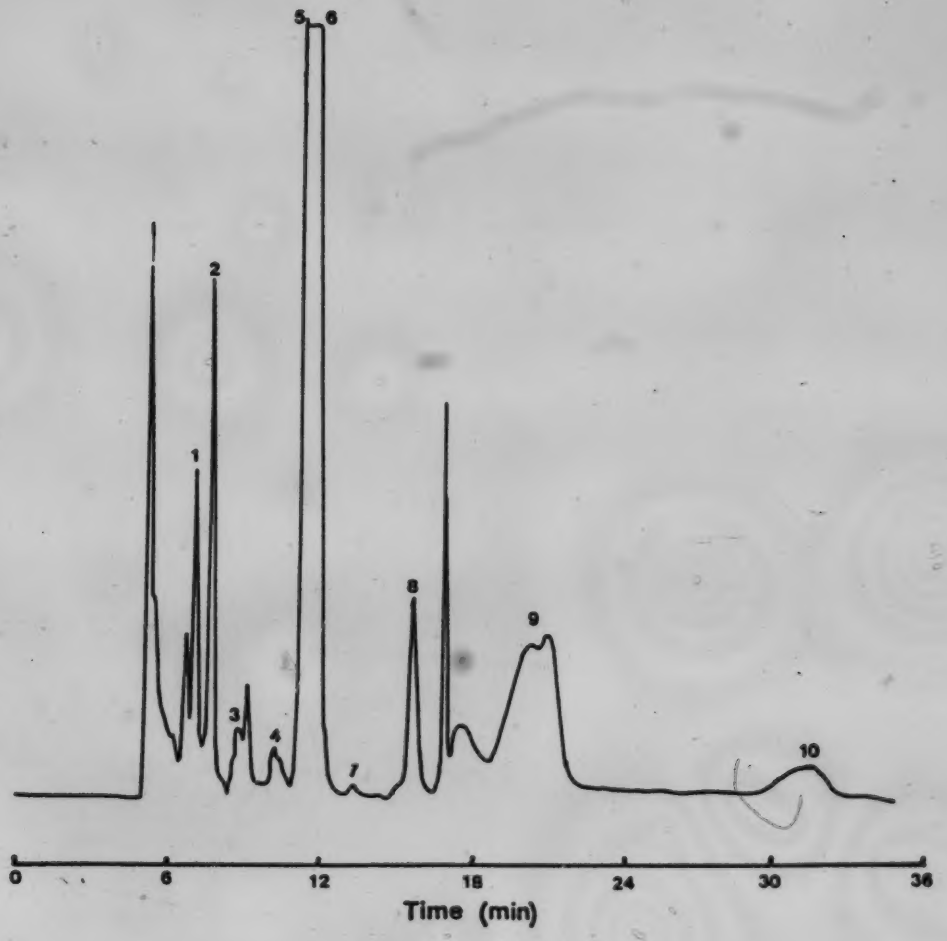

Figure 7.12. Liquid chromatogram of organic acids present in yogurt.
Yogurt was produced in the Edmonton plant of NADP from a blend of whole and skim milks and skim milk powder and gelatin as a stabilizer. Acidification was achieved with lactic starters *L. bulgaricus* and *S. thermophilus*. The fat and moisture contents of the product were 2.0 and 86.3%, respectively. Peak 9 (butyric acid) coeluted with a polypeptide.

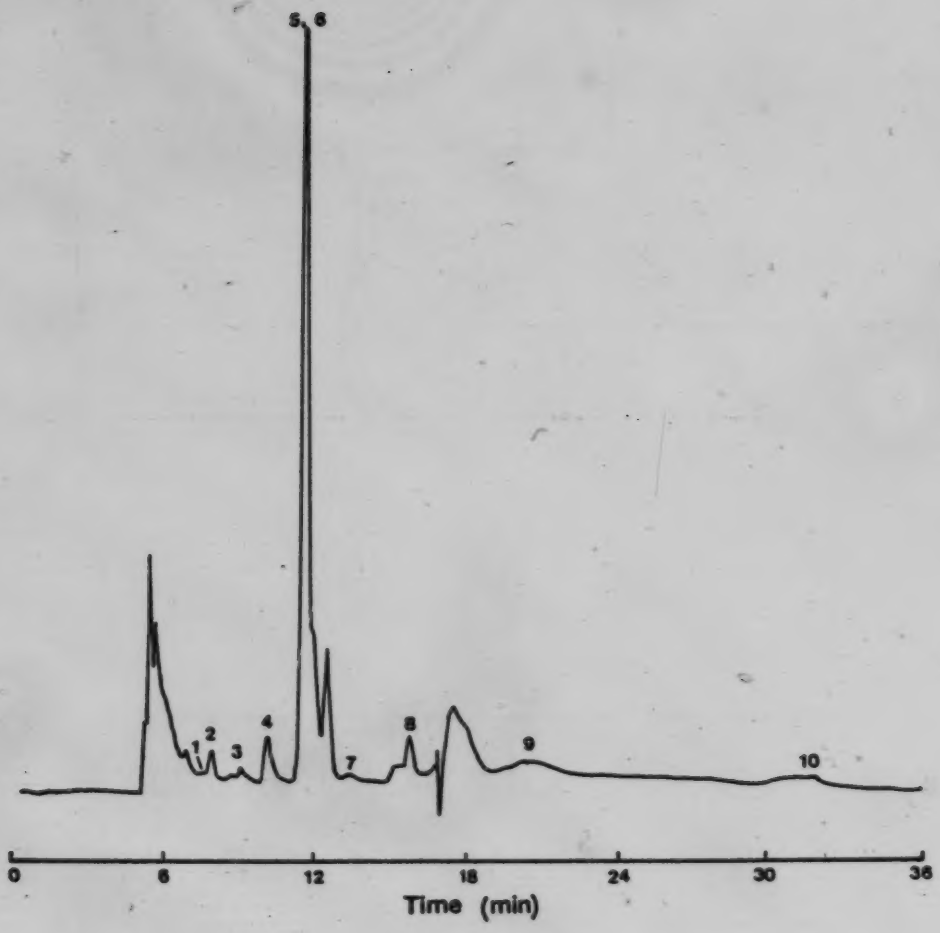

Figure 7.13. Liquid chromatogram of organic acids present in Camembert cheese.
The cheese was produced at Neapolis Dairy Products Ltd., Didsbury, Alta, from whole pasteurized milk by addition of lactic starter culture, rennet and the white mold *Penicillium candida*. The growth of mold was promoted by turning the cheese daily during the ripening period of 3-5 weeks, initially at 20°C (RH, 40), then 23°C (RH, 50-60) and 2°C (RH, 30). The cheese was then wrapped in Al-foil and stored in the cold. The fat and moisture contents of the product were 22.5 and 55.2%, respectively.

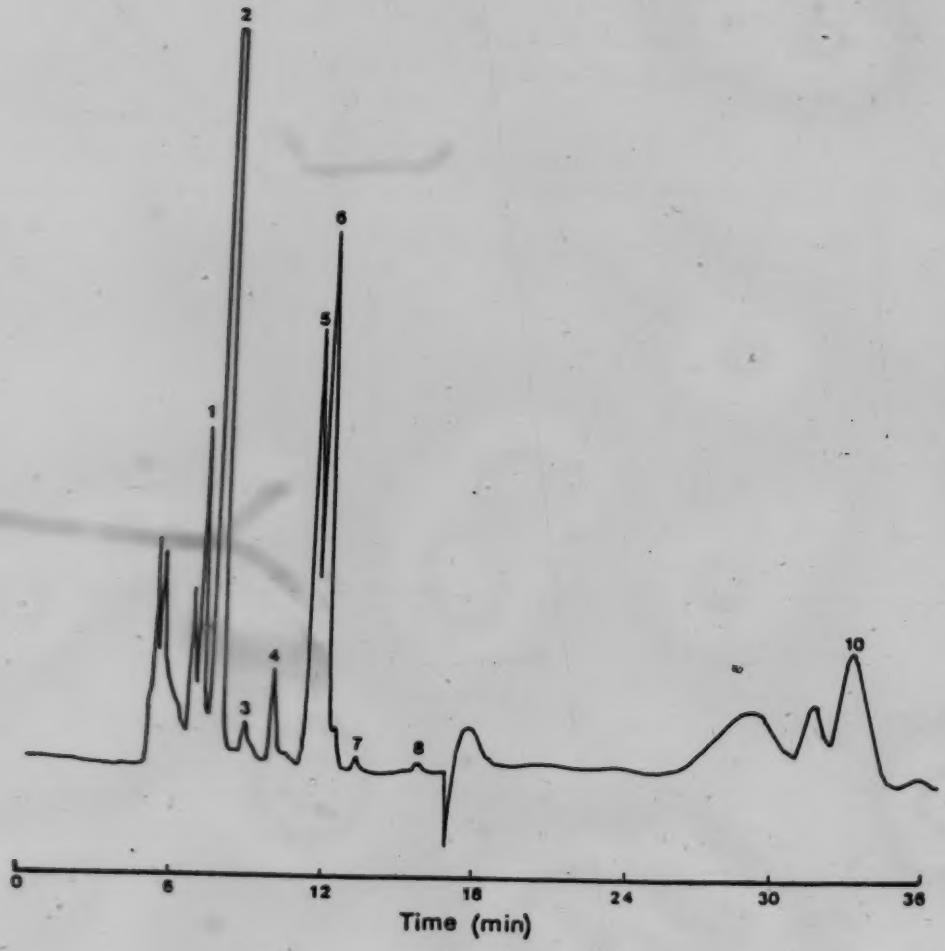

Figure 7.14. Liquid chromatogram of organic acids present in commercial cottage cheese.
Cottage cheese was manufactured by addition of a lactic starter culture followed by rennet treatment of skim milk to which skim milk powder and calcium chloride were added. Then the curd was salted and creamed to 20% BF. For details see Experimental. The moisture content of the product was 72.6%.

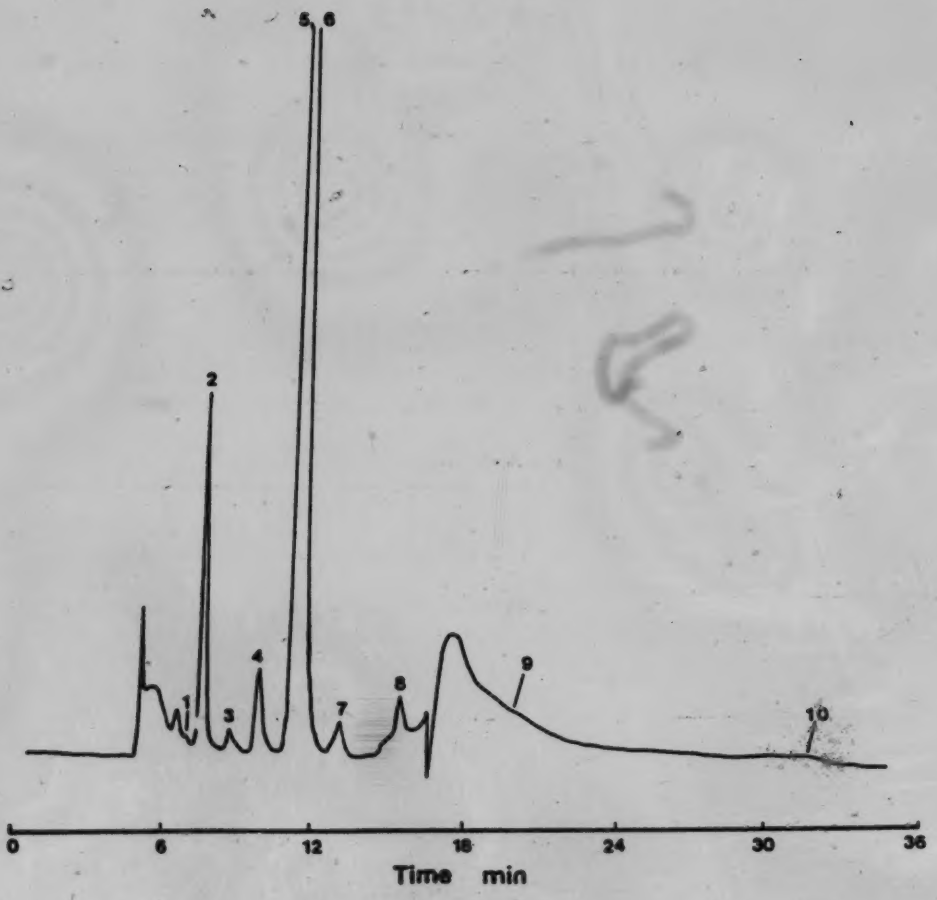

Figure 7.15. Liquid chromatogram of organic acids present in cheddar cheese.
Cheddar cheese samples were collected at the cheese plant in Glenwood, Alberta and after a 6-month ripening period. For details see Experimental. The fat and moisture contents of the product were 33.5 and 36.5%, respectively.

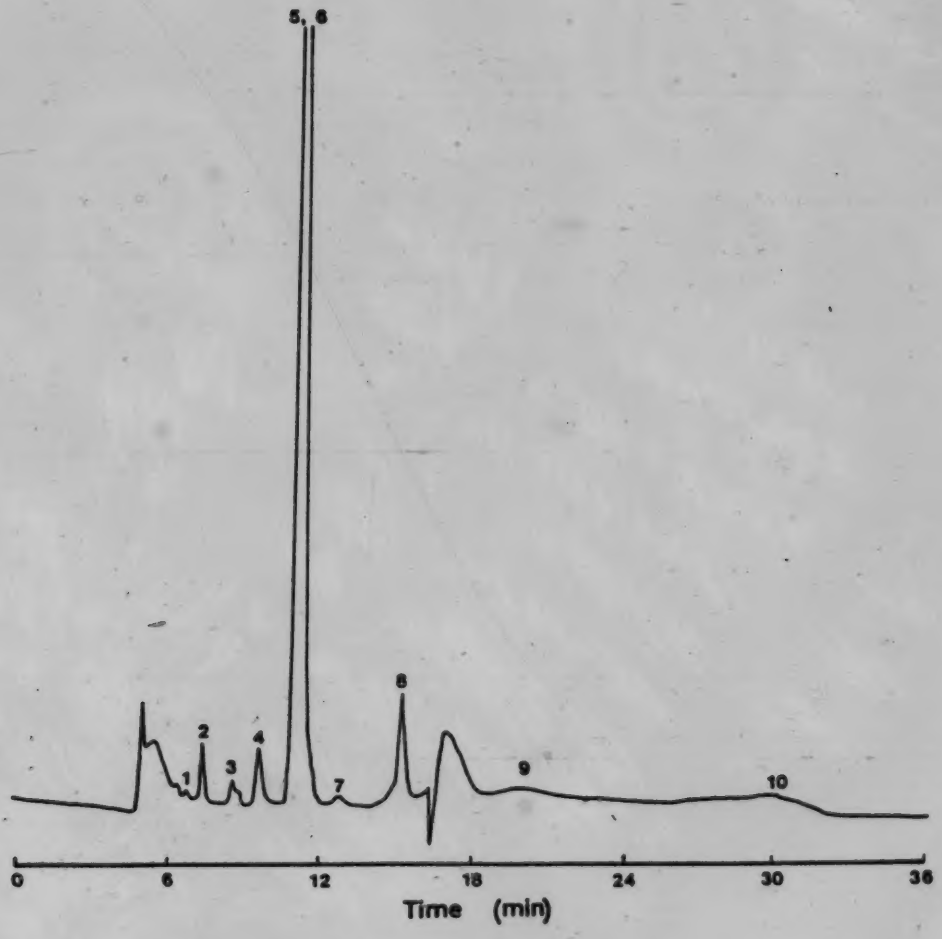

Figure 7.16. Liquid chromatogram of organic acids present in pressed Mozzarella cheese.
The cheese sample was collected at Alberta Cheese Co., Calgary, after a 4-week ripening period. In the production, the floating curd and whey were cooked at 38°C, the whey was drained, and the curd washed and pressed in rigid molds overnight. For details see Experimental. The fat and moisture contents of the product were 20.0% and 45.0%, respectively.

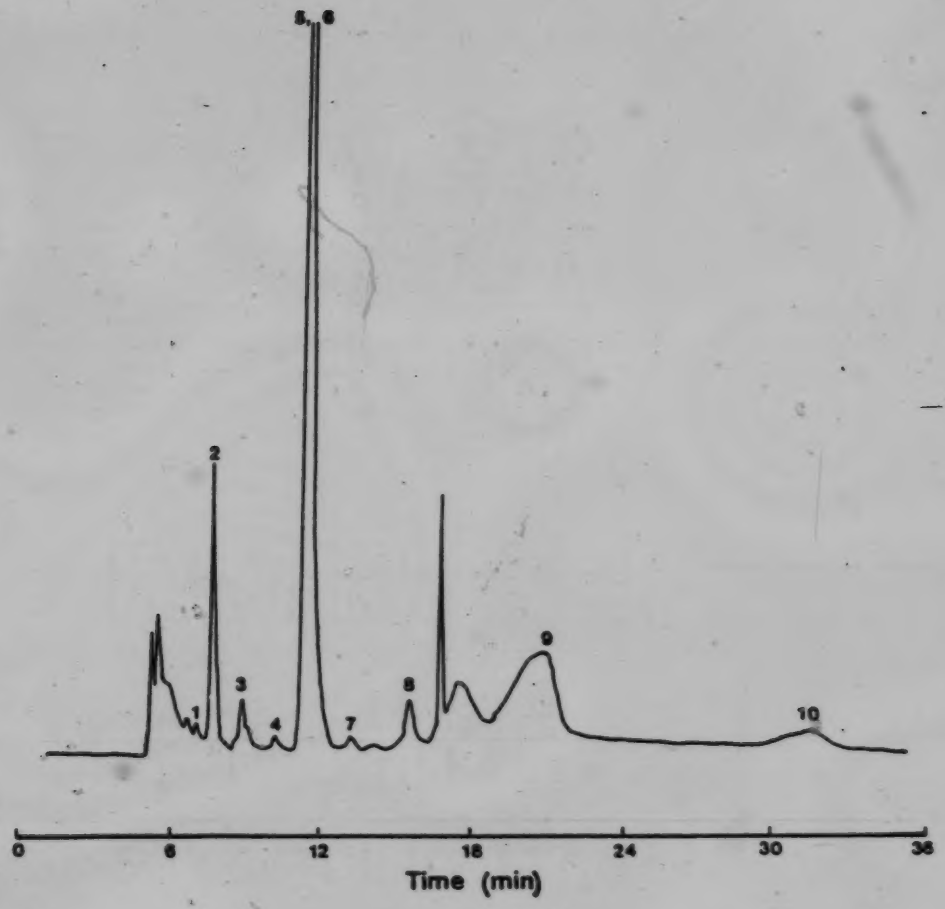

Figure 7.17. Liquid chromatogram of organic acids present in stretched Mozzarella cheese.
The cheese sample was collected at Alberta Cheese Co., Calgary. The cheese was produced similarly to Mozzarella pressed cheese with the exception of curd cooking at 39°C, slow drainage of whey, then pressing the curd into mats, chopping it into small pieces, then stretching it in hot water at 73°C. The plastic curd was then shaped in rigid molds, cooled and salted overnight in 20% brine and ripened for two weeks. The fat and moisture contents of the product were 15.0 and 52.0%, respectively.

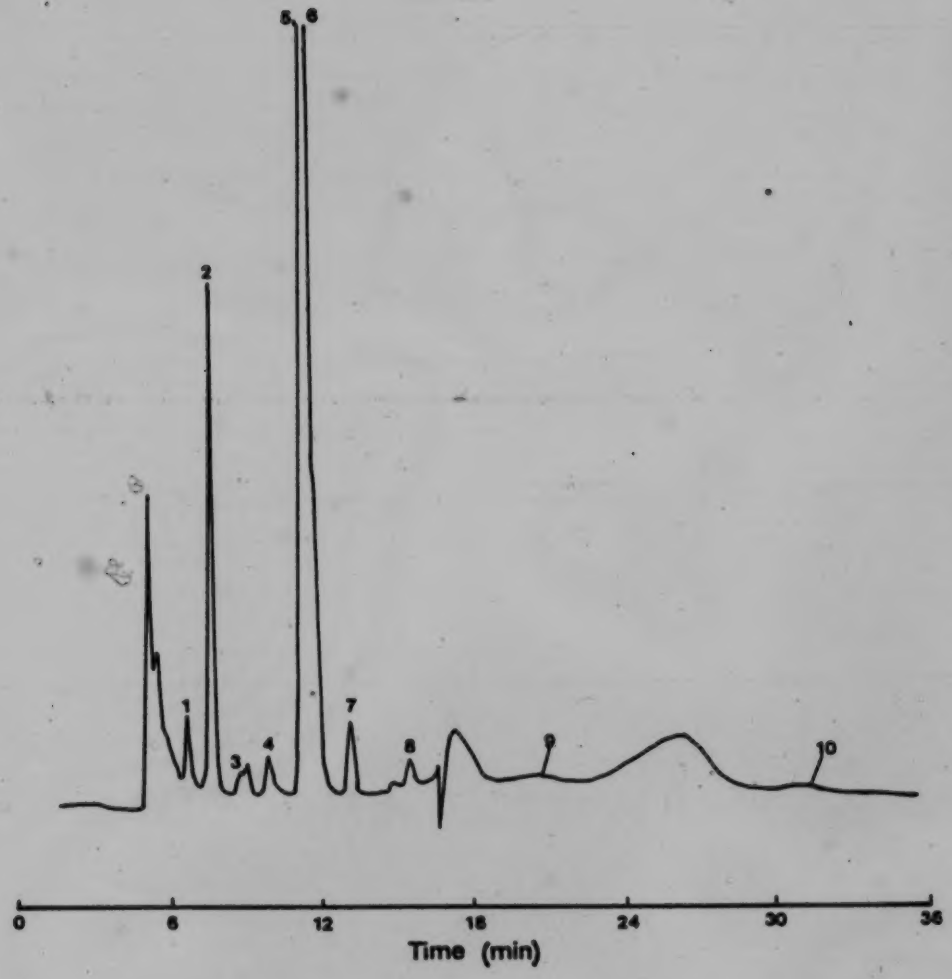

Figure 7.18. Liquid chromatogram of organic acids present in quark.
Quark was produced at Neapolis Dairy Products Ltd., Didsbury, Alta, from whole pasteurized milk by addition of lactic starter culture; the curd was separated from whey by pressing with a perforated screen. The fat and moisture contents of the product were 13.5 and 72.5%, respectively.

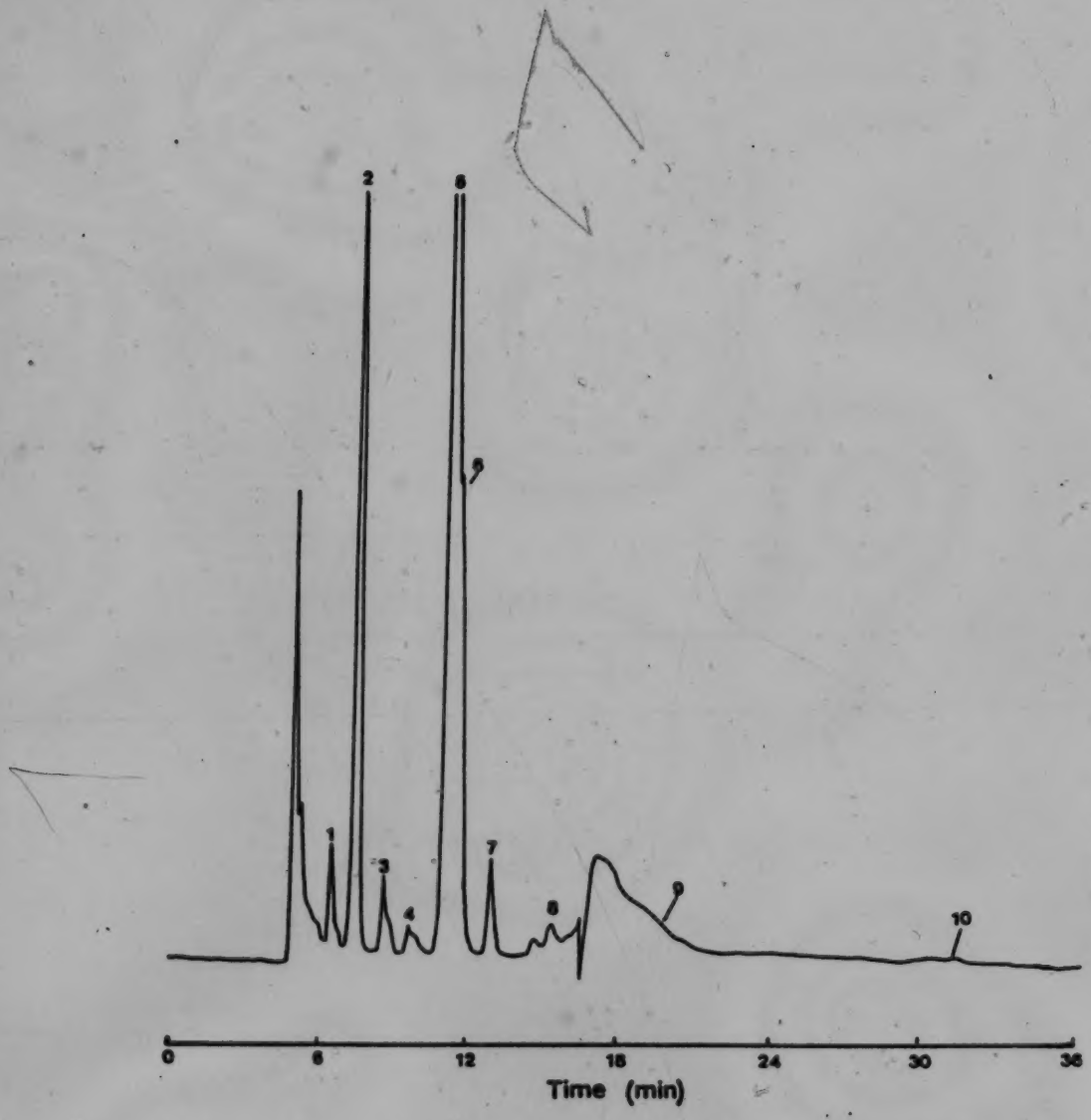

Figure 7.19. Liquid chromatogram of organic acids present in quark whey.
The whey was collected during quark manufacturing at Neapolis Dairy Products Ltd., Didsbury, Alta. See Figure 6.19. The solids content of the whey was 5.6%.

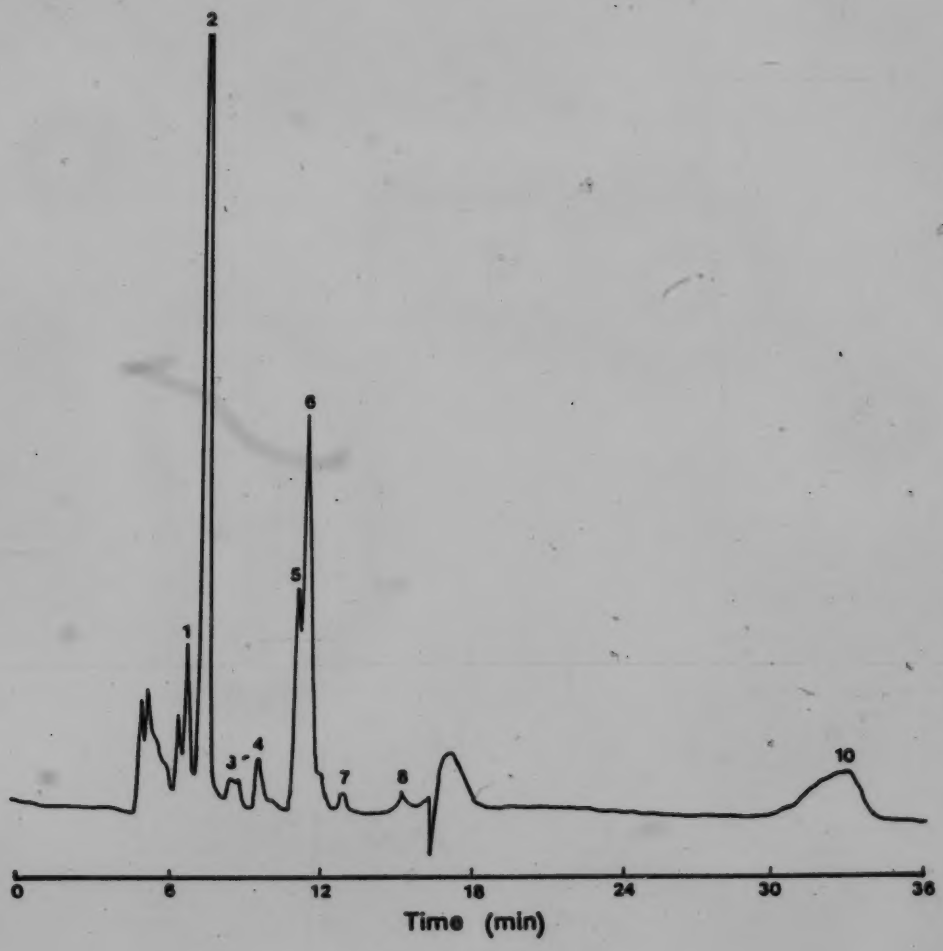

Figure 7.20. Liquid chromatogram of organic acids present in Ricotta cheese.
The cheese sample was collected at Alberta Cheese Co., Calgary. The cheese was produced from Mozzarella whey to which milk, cream and sodium chloride were added prior to acidification with a blend of Ricotta whey (from a previous batch process) and citric acid. The separated curd was drained and then packed. The fat and moisture contents of the product were 0.5 and 75.0%, respectively.

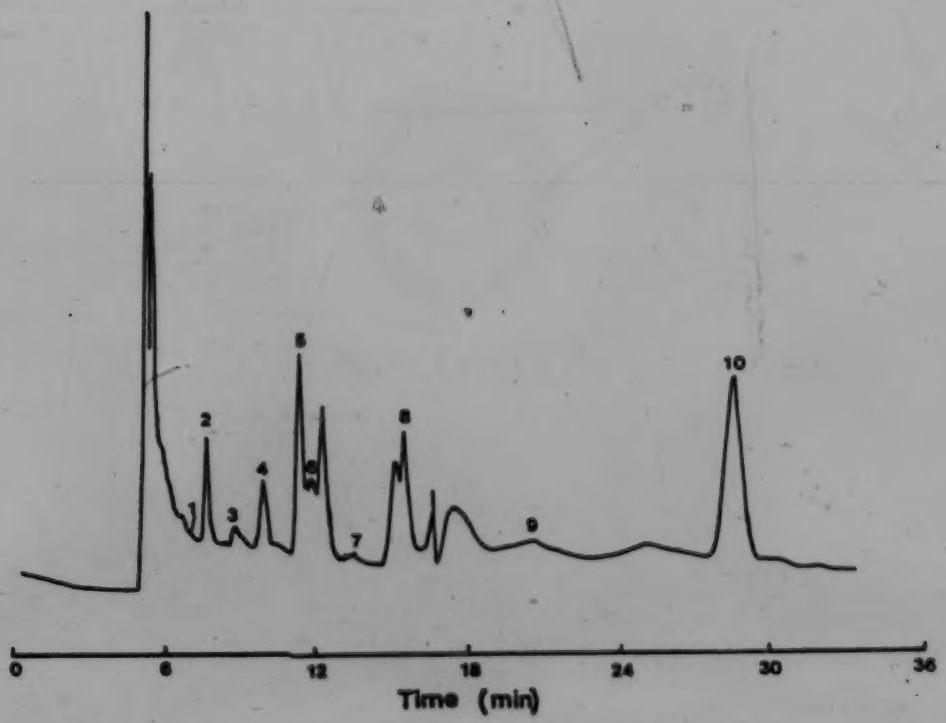

Figure 7.21. Liquid chromatogram of organic acids present in Danish Camembert.

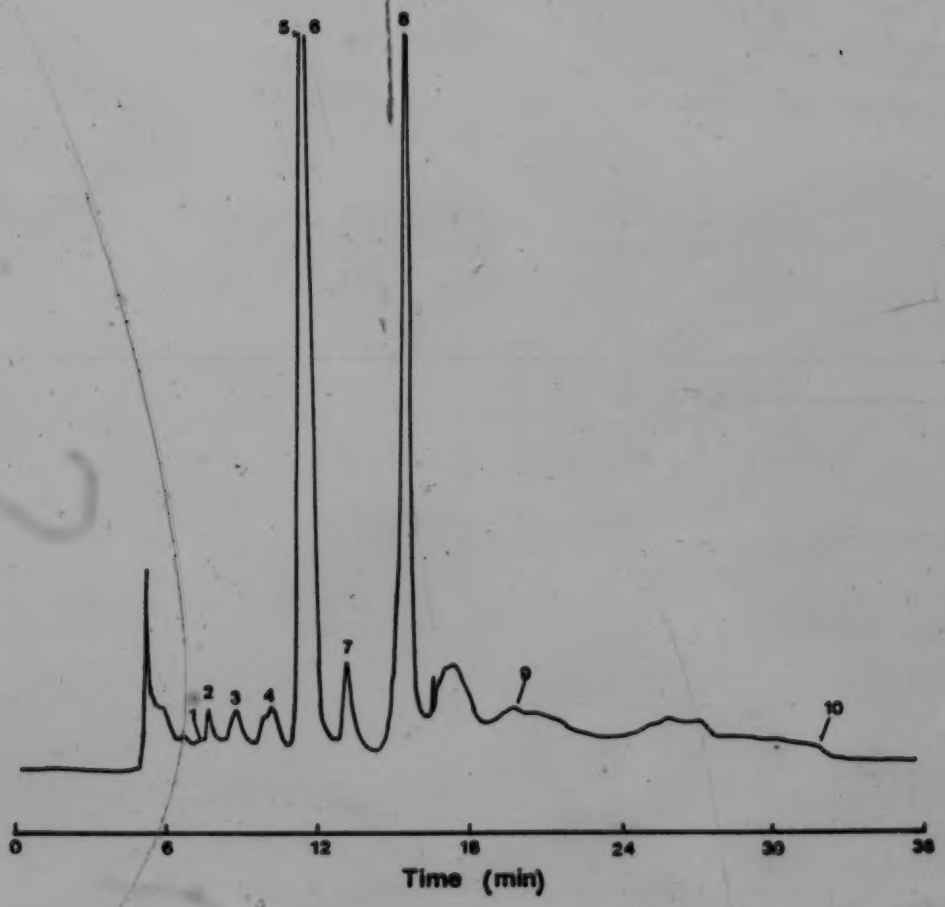

Figure 7.22. Liquid chromatogram of organic acids present in Danish Danablue.

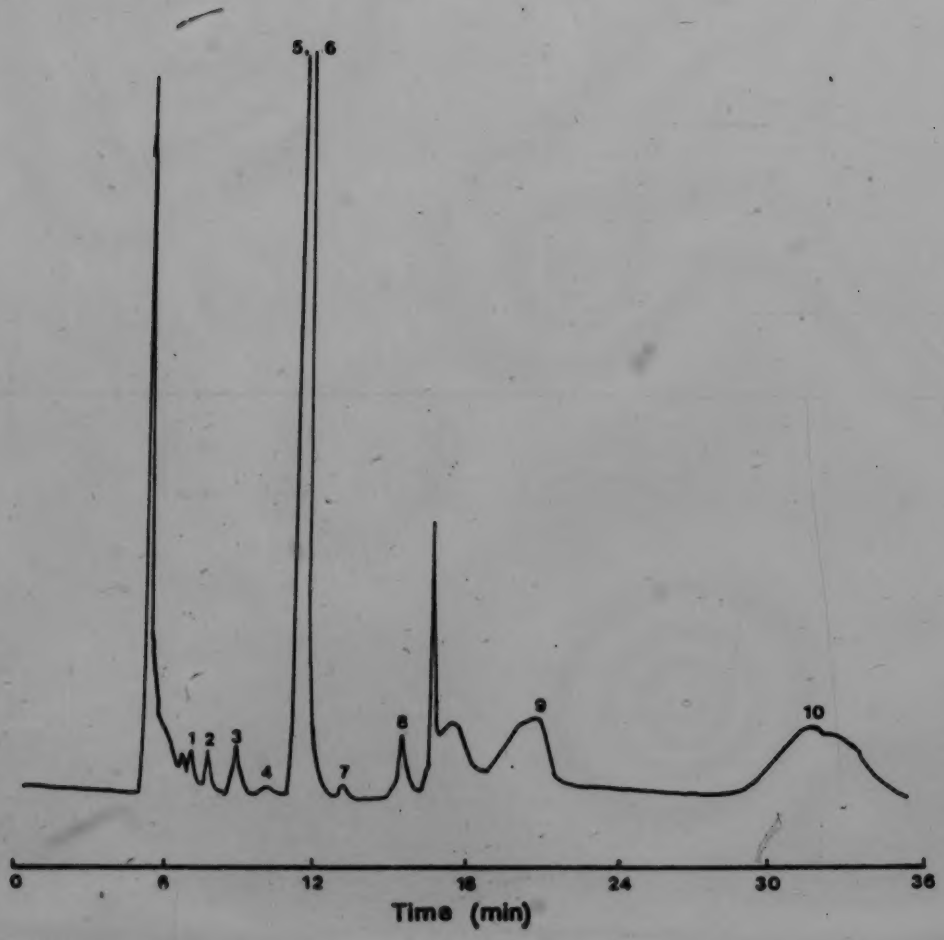

Figure 7.23. Liquid chromatogram of organic acids present in Danish Feta.

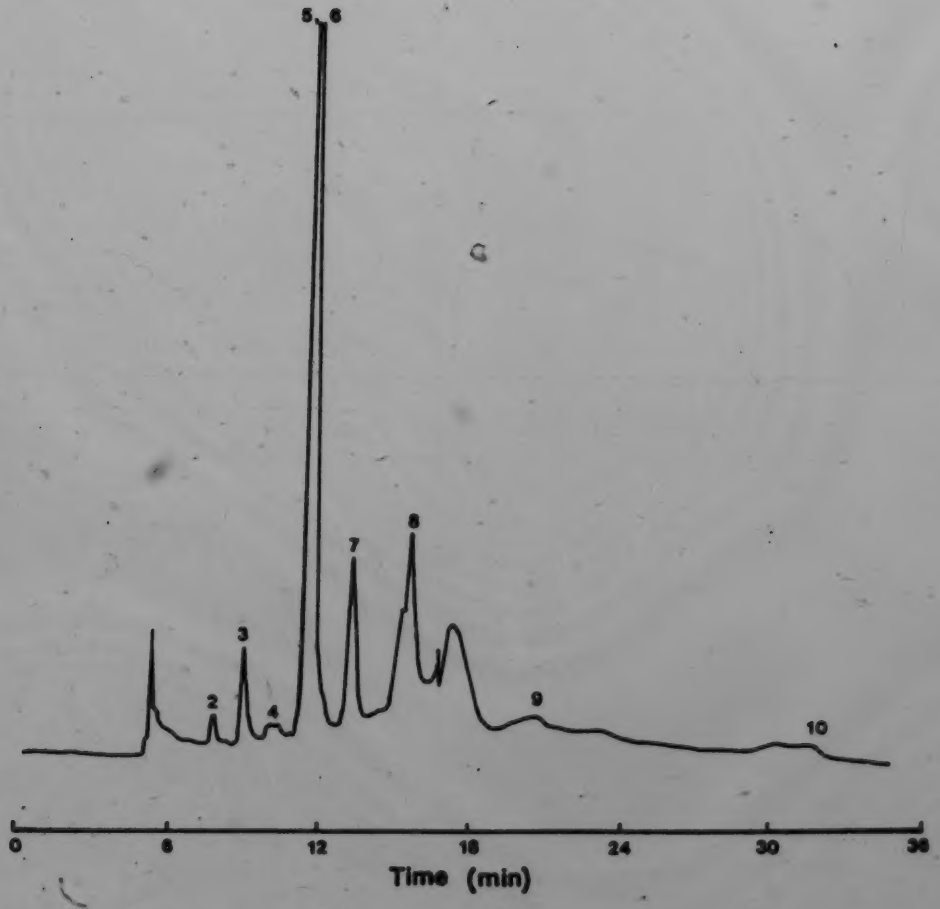

Figure 7.24. Liquid chromatogram of organic acids present in Danish Havarti.

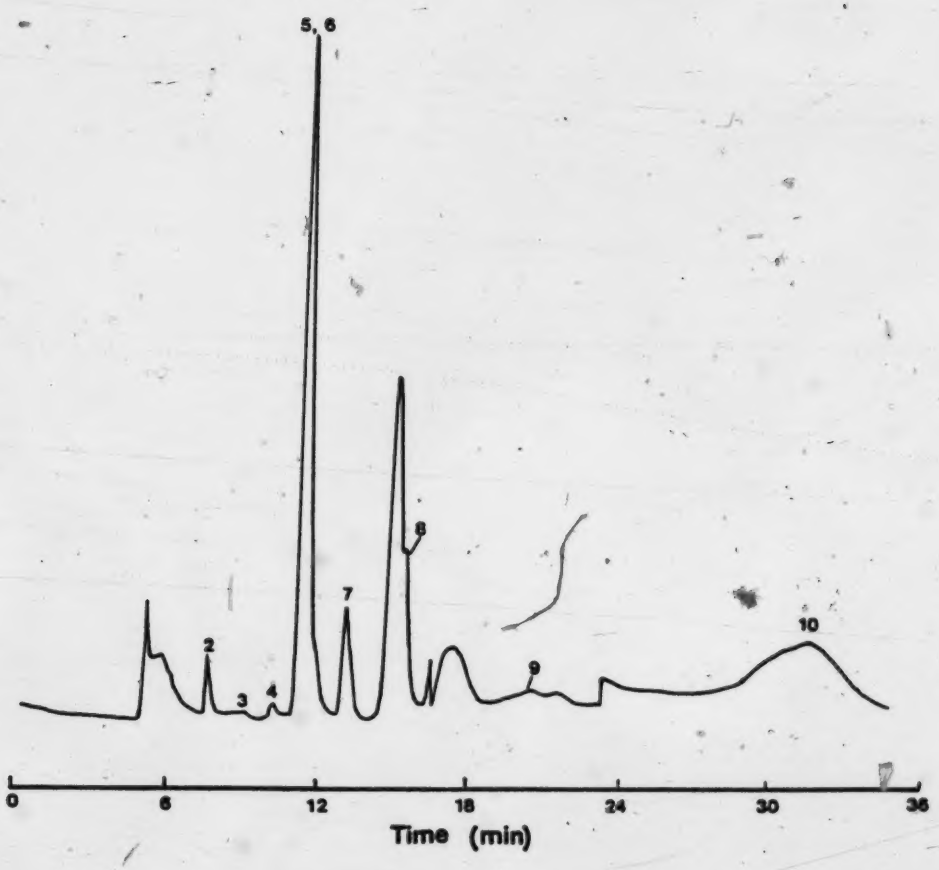

Figure 7.25. Liquid chromatogram of organic acids present in Danish Samsoe.

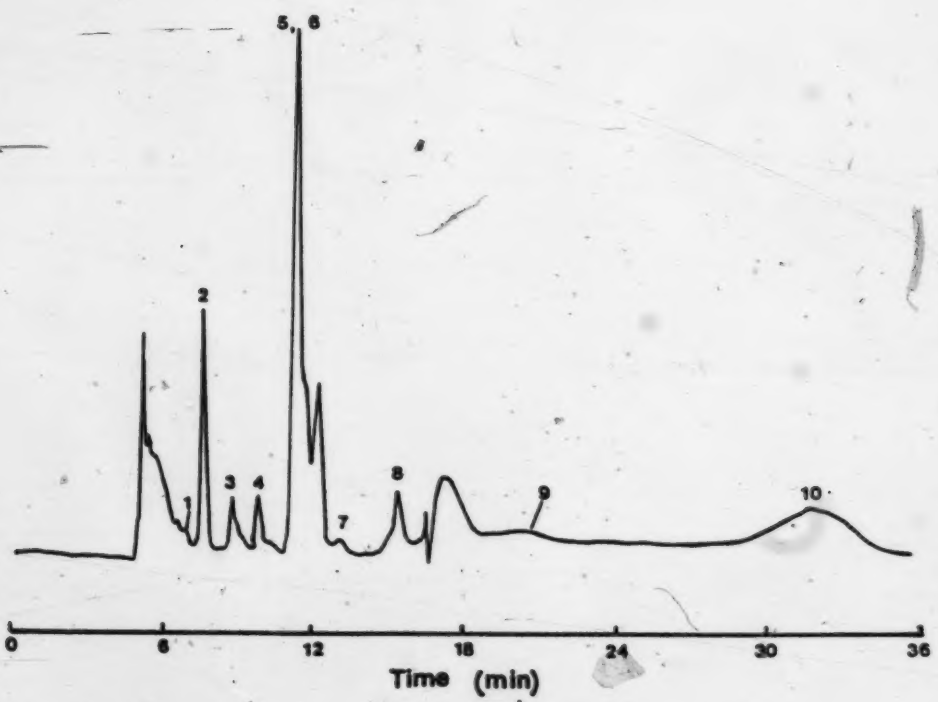

Figure 7.26. Liquid chromatogram of organic acids present in French Brie.

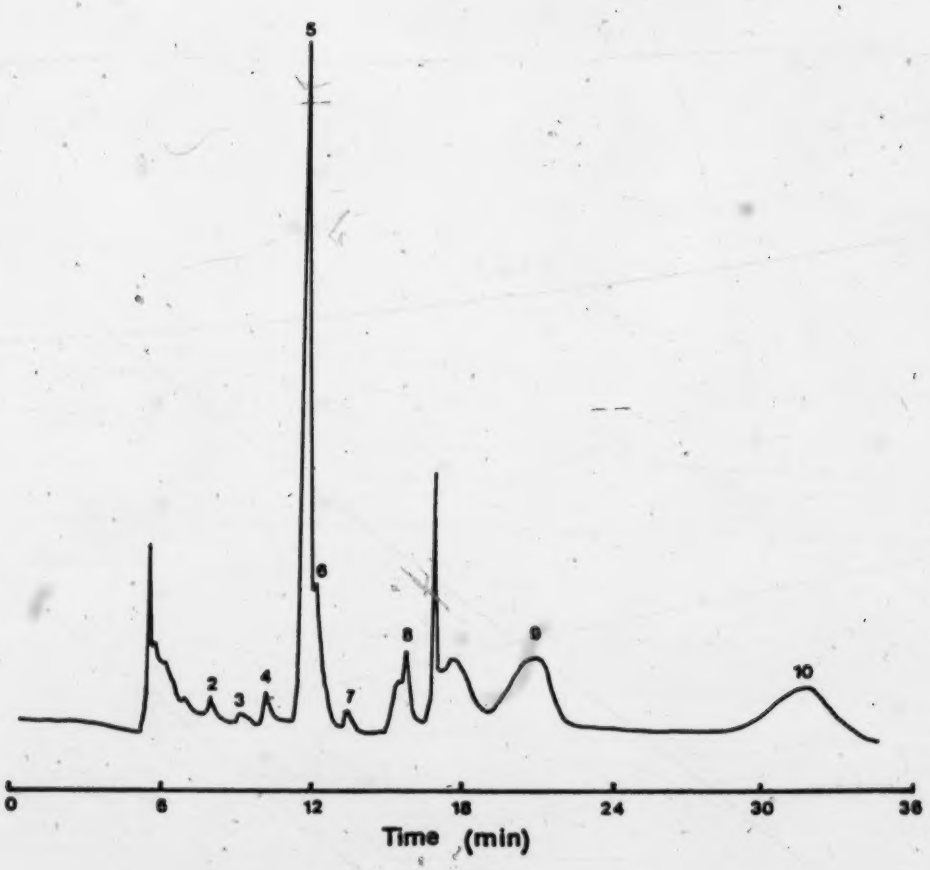

Figure 7.27. Liquid chromatogram of organic acids present in French Camembert.

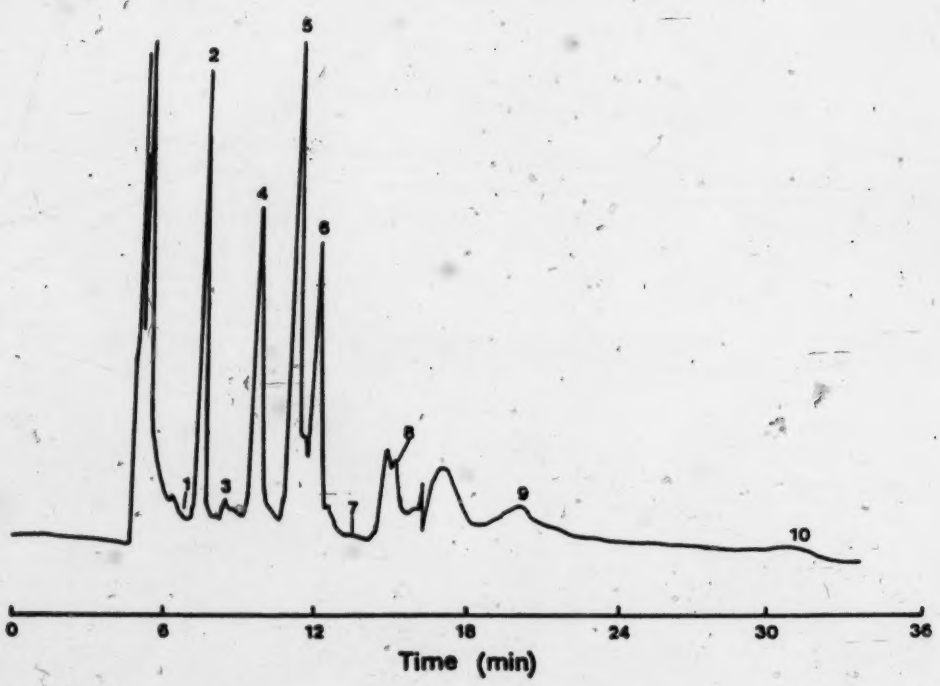

Figure 7.28. Liquid chromatogram of organic acids present in French Marcillat.

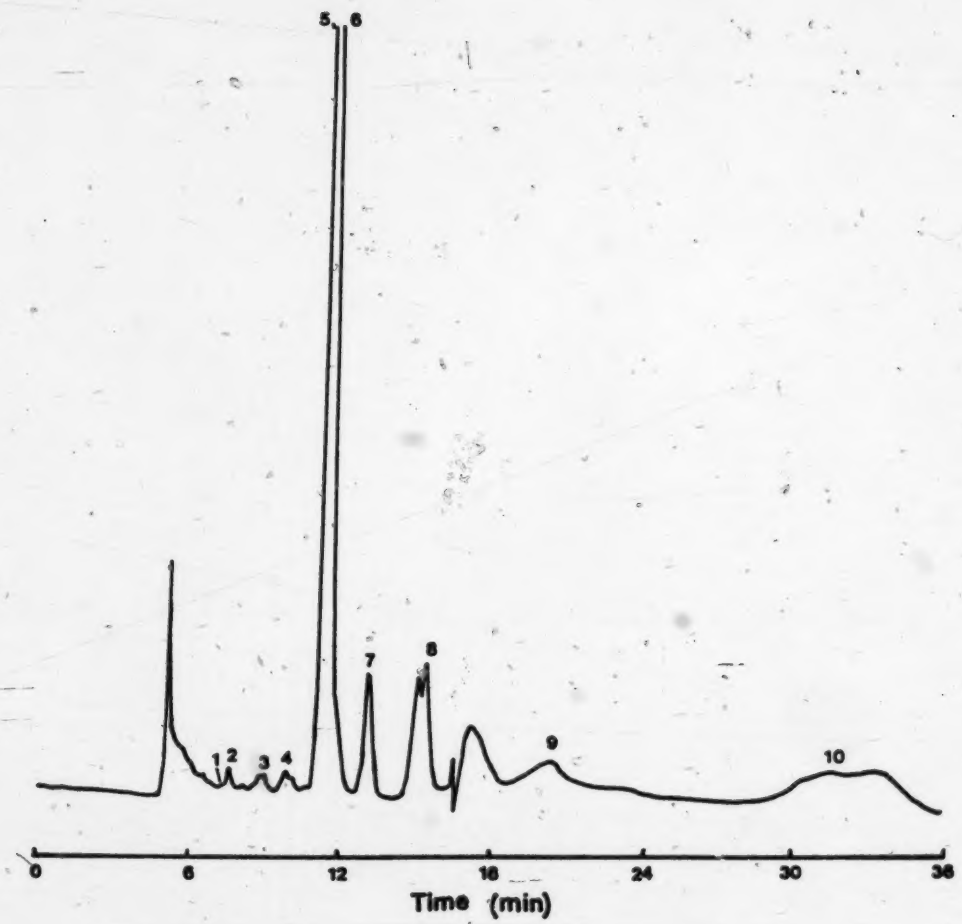

Figure 7.29. Liquid chromatogram of organic acids present in French Port Salut.

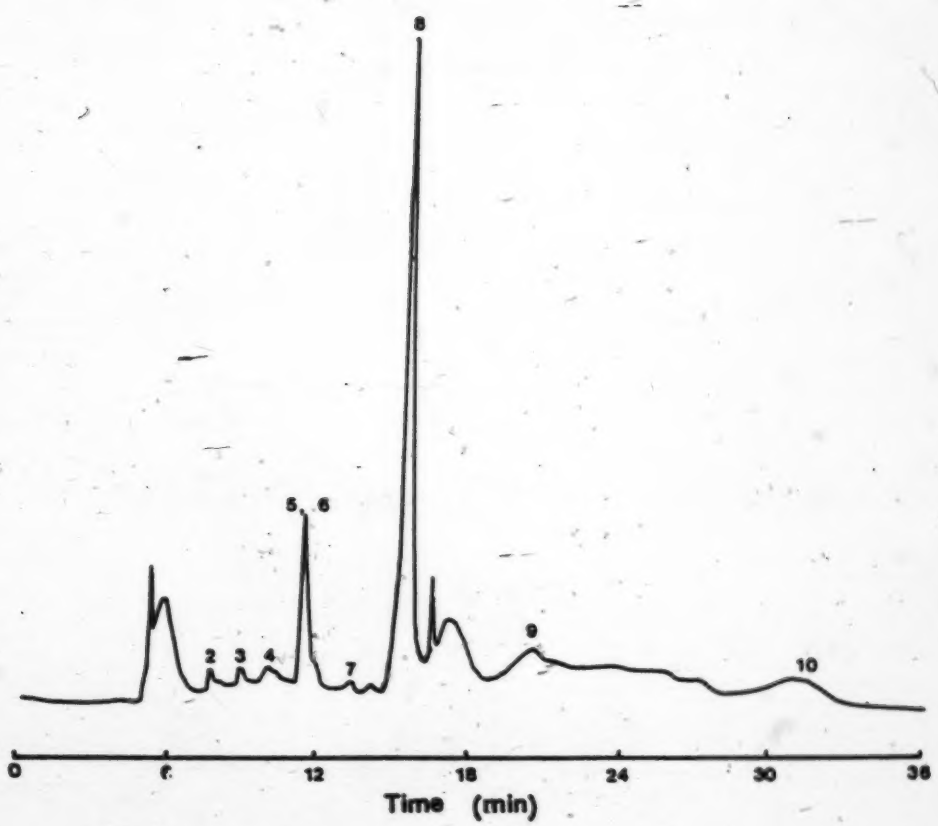

Figure 7.30. Liquid chromatogram of organic acids present in French Roquefort.

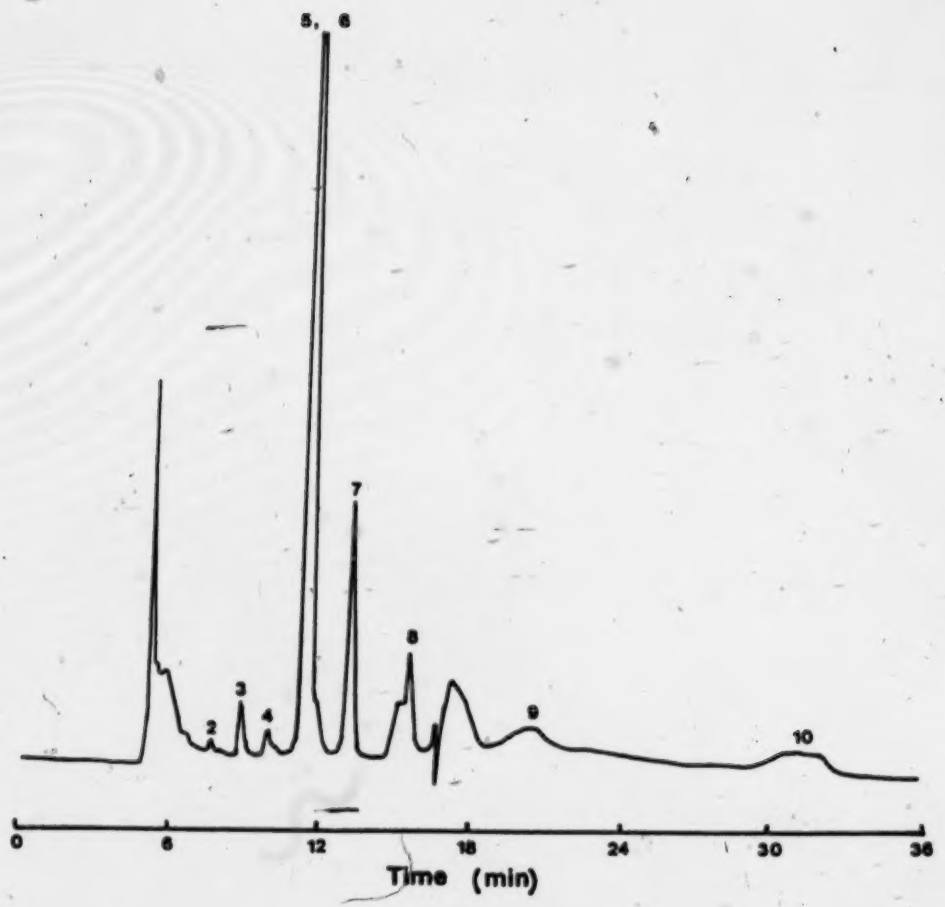

Figure 7.31. Liquid chromatogram of organic acids present in Dutch Edam.

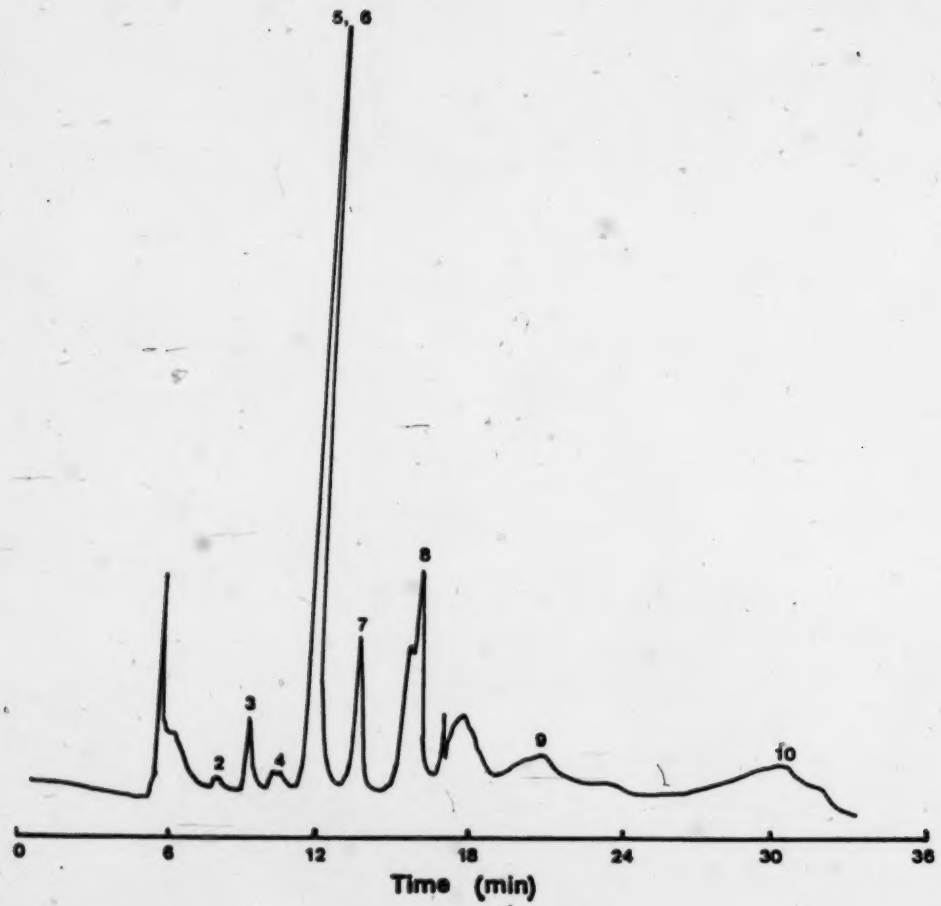

Figure 7.32. Liquid chromatogram of organic acids present in Dutch Gouda.

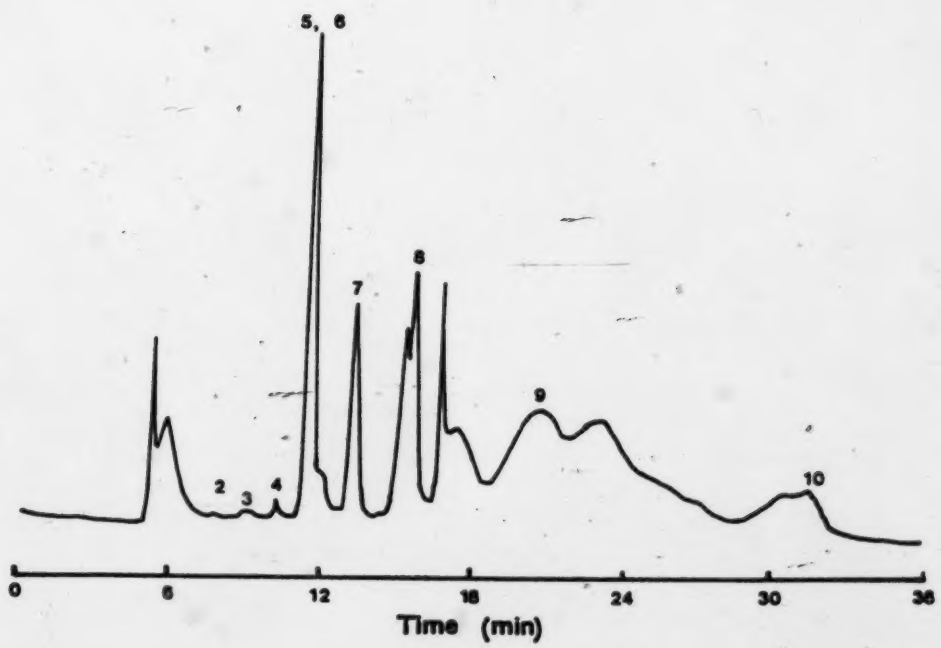

Figure 7.33. Liquid chromatogram of organic acids present in Swiss Appensell.

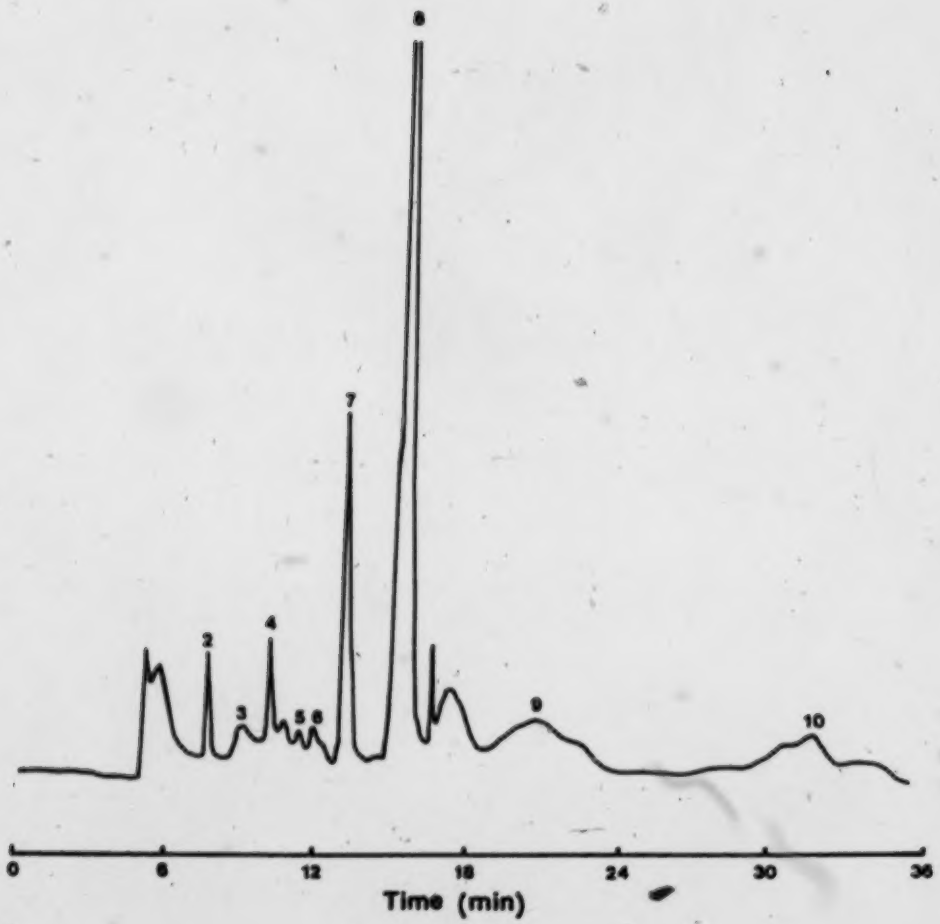

Figure 7.34. Liquid chromatogram of organic acids present in Swiss Emmental.

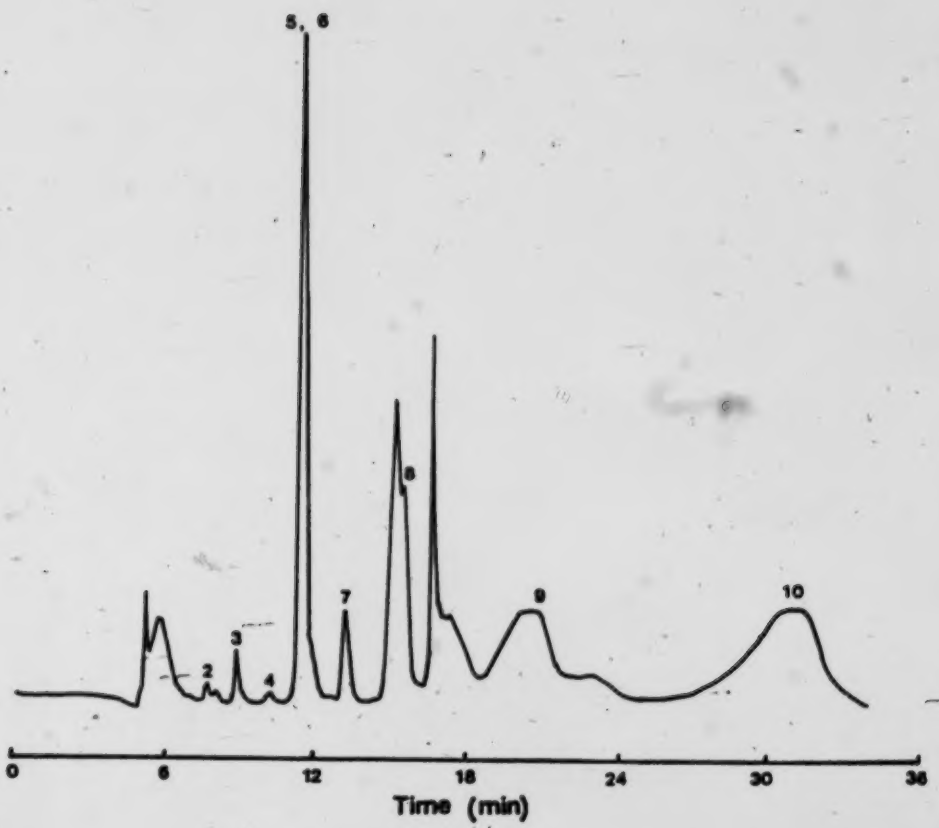

Figure 7.35. Liquid chromatogram of organic acids present in Swiss Gruyère.

VITA

NAME: Marija Skerlj

PLACE OF BIRTH: Belgrade, Yugoslavia

DATE OF BIRTH: August 21, 1960

PERMANENT ADDRESS: Linhartova 84
61000 Ljubljana
Yugoslavia

POST-SECONDARY EDUCATION AND DEGREES:

Edvard Kardelj University of Ljubljana
Faculty of Biotechnology
Department of Food Technology
1982-1983
Professional Engineer of Food Technology
Diploma work title: Buttermilk Analysis by Milco-Scan

COMMENCEMENT OF M.Sc. PROGRAM:

September 1983
Department of Food Science
University of Alberta
Food Chemistry
(thesis topic: Organic Acids in Dairy Products)

RELATED WORK EXPERIENCE:

1983-1984
Demonstrator, FdSc 506 (Cereal and Oil Seed Processing)
Department of Food Science, University of Alberta

1984-1985
Teaching Assistant, FdSc 402 (Quality Control)
Department of Food Science, University of Alberta

CONFERENCES, CONGRESSES AND MEETINGS ATTENDED:

Alberta Dairymen's Association, Annual Meeting
Red Deer, AB, March 14, 1984

Canadian Institute of Food Science and Technology
Annual Conference, Vancouver, BC, May 27-30, 1984

Institute of Food Technologists, Annual Meeting
Anaheim, CA, June 10-13, 1984

International Congress on Engineering in Food
Edmonton, AB (Student Volunteer), July 7-10, 1985

NON-ACADEMIC ACTIVITIES:

Alternate Graduate Student Representative, 1984-1985
Department of Food Science, University of Alberta